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Acetohydroxamic Acid - A Competitive Inhibitor of Urease from Soybean "*Glycine max*"

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Abstract: *The acetohydroxamic acid (AHA), a potent inhibitor of urease, inhibits soybean urease competitively and reversibly. The I_{50} and K_i value for AHA were 900 μM and 0.053 mM, respectively at pH 7.0, 37 °C. The variation in pH over the pH 6 - 9 affected K_i and therefore binding of AHA in the active site. The affinity of AHA for the active site decreases with lowering of pH (below the pK_a value of AHA i.e. 8.7). This behaviour is consistent with the deprotonated AHA acting as a nucleophile or the inhibitory species. The time-dependent inhibition studies were performed at two different concentrations of AHA and the biphasic kinetics was revealed with almost equal amplitudes (50% each) for fast and slow phases. The values of rate constants were 0.1642 ± 0.0013 min^{-1} (fast phase); 0.0123±0.0012 min^{-1} (slow phase) at 0.10 mM AHA and 0.2379±0.0017 min^{-1} (fast phase); 0.0153±0.0010 min^{-1} (slow phase) at 0.15 mM AHA. These studies established the asymmetric nature of active sites, half being more reactive for AHA than the other half. The spectral studies showed a change in absorbance at the λ_{max} 414 nm, when urease was incubated with AHA, which was consistent with AHA binding to Ni^{2+} of active site.*

Keywords: *Urease, Acetohydroxamic Acid, Soybean, Molecular Asymmetry, Inhibition.*

I INTRODUCTION

Acetohydroxamic acid (AHA) is one of the inhibitors studied as the potential therapeutical drug for the treatment of ulcer caused by *H. pylori* and has also been suggested as a means of preventing the various related pathological conditions. Hydroxamic acids are the family of compounds presenting the strong chelating properties towards various metal ions (Kurzak et al., 1992). The coordination phenomenon is responsible for many biochemical actions in which naturally occurring hydroxamic acids participate. The compounds containing the hydroxamic group have been shown to inhibit activity of various metalloproteinases such as urease (Stemmler et al., 1995), oxidases (Ikeda-Saito et al., 1991), Alzheimer's Amyloid Precursor Protein asecretase (Parvathy et al., 1998) or zinc proteinases involved in cancers (Hajduk et al., 1997; Groneberg et al., 1999). Unsubstituted hydroxamic acids are organic ligands having the general formula RCONHOH; they act as good chelating agents for metal ions through the formation of five-membered chelate rings. One of the simplest hydroxamic acids is acetohydroxamic acid ($CH_3CONHOH$), which has been extensively studied by theoretical methods (Ventura et al., 1993; Bagno et al., 1994; Muñoz-Caro et al., 2000; Niño et al., 2000).

In present studies, the soybean urease was subjected to inhibition studies with AHA. All ureases, irrespective of their source, have similar structure of their active site and therefore exhibit identical catalytic mechanism. The soybean urease was chosen as a model system to study the AHA inhibition for potential applications in the treatment of various diseases. Also such investigations can have potential role in other industries like agriculture and environment to solve the problems such as air pollution and to prevent the NH_3 losses from the soil.

II MATERIALS AND METHODS

Chemicals

Acetohydroxamic acid (AHA), Bovine serum albumin (BSA), Tris and Urea (Enzyme grade) were from Sigma Chemicals Co., St. Louis, USA. Nessler's reagent, TCA (Trichloroacetic acid), Na_2HPO_4 and NaH_2PO_4 were purchased from HiMedia, Mumbai, India. All other chemicals were of analytical grade obtained from Merck or BDH, India. All the solutions were prepared in Milli Q (Millipore, USA) water.

Enzyme

The urease was purified from the mature seeds of soybean to apparent homogeneity by the method of Polacco and Havir (1979) with slight modifications.

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Urease Activity Assay

For routine assay of urease activity, the ammonia liberated in a fixed time interval while incubating the enzyme with saturating concentrations of urea, was determined by using Nessler's reagent as described earlier (Das et al., 2002). The yellow-orange colored solution was measured at 405 nm on an ATI-Unicam UV-2 Spectrophotometer. The amount of NH_3 liberated in the reaction mixture was calculated by calibrating the Nessler's reagent with standard NH_4Cl solution. One enzyme unit is defined as the amount of urease required to liberate 1 µmol of ammonia per min under our test conditions (0.1 M urea, 0.05 M Tris-acetate buffer, pH 7.0, 37°C).

Protein Estimation

Protein content was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Inhibition Studies

The stock solution of AHA was prepared in 0.05 M Tris-acetate buffer, pH 7.0 and diluted with the same buffer as required. The activity assay was performed with varying concentrations of AHA in two different concentrations of urea (0.1 M and 0.3 M) under normal assay conditions. The K_i value was determined from Dixon plot. Further, in order to study the effect of pH on K_i, the pH was varied (pH 6.0 to 9.0) and the activity was determined in the presence of AHA as described earlier. The phosphate buffer (pH 6.0-7.0) and Tris-acetate buffers (pH 7.0-9.0) were used to cover the specified pH range. For time-dependent inhibition studies, the urease (2.28 µg/ml) was incubated with 0.10 or 0.15 mM AHA in 0.1 M Tris-acetate, pH 7.6 and aliquots withdrawn at different time intervals were assayed for residual activity. The values of rate constant and amplitude were calculated as described previously (Kayastha and Gupta, 1987). Furthermore, the urease was incubated with 1 mM AHA for 25 min at 37°C in the absence of urea. The activity assay was performed with AHA inhibited and untreated urease after incubating for the specified period. The AHA inhibited urease was subsequently dialyzed overnight against 0.1 M Tris-acetate; pH 7.6 at 4°C and activity was determined after 24 h.

Spectral Studies

The urease (20 mg/ml) was incubated with 12 mM AHA in 0.05 M Tris acetate buffer, pH 7.0 at 37°C for 25 min. The absorption spectra were recorded for native and AHA treated urease separately on an ATI-UNICAM UV-2 Spectrophotometer. Control spectrum of enzyme and AHA as well as the difference spectrum were also recorded.

Analysis of the Kinetic Data

With time-dependent inhibition, the data was plotted as log % residual activity versus time. The time-courses of inhibition was found to consistent with Eq. (1) and therefore the data was processed and analyzed in accordance with the following equation:

$$A_t = A_{fast}\, e^{-k}\text{fast}^{\cdot t} + A_{slow}\, e^{-k}\text{slow}^{\cdot t} \qquad (1)$$

where A_t is the fraction residual activity at time t, A_{fast} and A_{slow} are amplitudes (expressed as percent of the starting activity) and k_{fast} and k_{slow} are the rate constants of the fast and the slow phases, respectively. Initial estimates of the rate constants and amplitudes were obtained from the semi-log plots as described earlier (Kayastha and Gupta, 1987).

III RESULTS AND DISCUSSION

Effect of AHA on the Activity of Urease

The hydroxamic acids were shown to be potent inhibitor of jack bean urease (Kobashi et al., 1962). Among them the AHA is most extensively studied hydroxamate. The various parameters of urease inhibition by AHA were investigated. Earlier the effects of AHA on urease have been reported by various workers from bacterial and plant sources (Kobashi et al., 1962; Dixon et al., 1975; Srivastava et al., 2002; Prakash and Upadhyay, 2004).

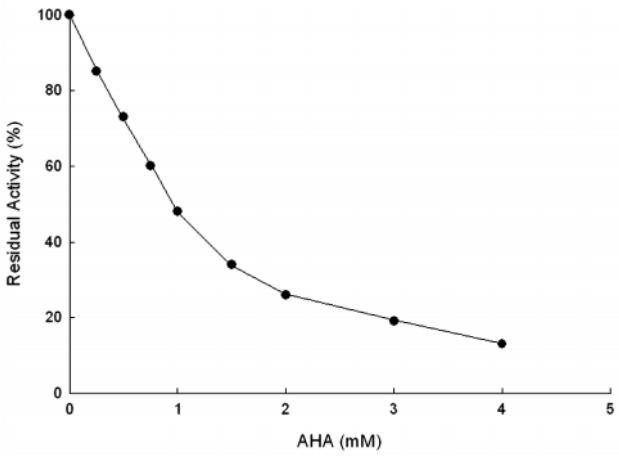


Figure 1 : Effect of AHA on the Activity of Soybean Urease. Suitably Diluted Urease (0.87 µg/ml) was Incubated for 10 min in the Presence of Varying Concentrations of AHA under Normal Assay Conditions

The urease was assayed with varying concentrations of AHA (0.25–4 mM) and it was observed that 50 % loss in activity (I_{50}) was obtained at 900 µM AHA (Figure. 1). Most investigators have shown the competitive nature of AHA inhibition and reported

the I_{50} values in the micro molar range for the plant ureases. The I_{50} values for pigeonpea urease (Srivastava et al., 2002) and watermelon (Prakash and Upadhyay, 2004) were reported at 250 µM and 700 µM, respectively. Most investigators have shown the competitive nature of AHA inhibition and reported the I_{50} values in the micromolar range for the plant ureases. However, I_{50} value alone does not provide insight into inhibitory mechanism.

Determination of Inhibition Constant (K_i) for AHA

The AHA being a metal chelator has been shown to form a stable bidentate complex with Ni^{2+} ions, and other ions in model system (Dixon et al., 1980). Hydroxamic acids and their derivatives have been reported to inhibit urease by competitive, non-competitive, mixed, or irreversible mechanism (Mobley et al., 1995). The activity assay was performed with 0.1 M or 0.3 M urea in presence of varying concentration of AHA (0.1–0.9 mM). The K_i was determined by Dixon method and the value was found to be 0.053 mM at optimum conditions (Figure 2). The competitive nature of AHA inhibition is quite evident from the pattern of the plot. Also it may be noted that when urea concentration was raised, an increase in the activity was observed. The K_i values for jack bean urease (Dixon et al., 1980), pigeonpea urease (Srivastava et al., 2002) and water melon urease (Prakash and Upadhyay, 2004) have been reported at 19.8 µM, 41 µM and 250 µM, respectively. The K_i value (53 µM) determined for soybean urease is quite close to that from other plant sources.

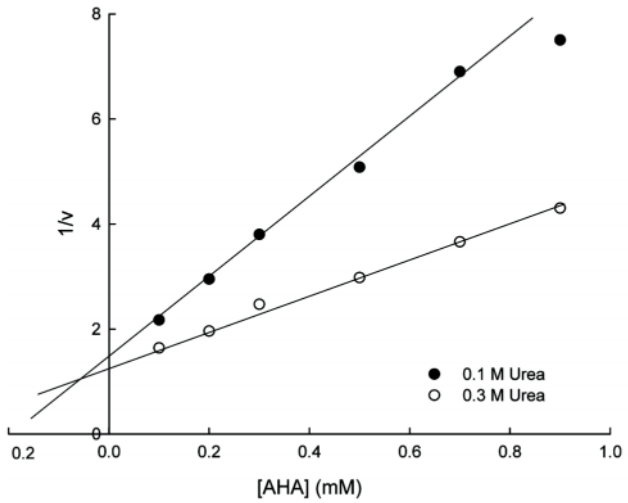


Figure 2 : Determination of K_i value for AHA Inhibition of Soybean Urease. The Enzyme (0.87 µg/ml) was Incubated with Varying Concentrations of AHA (0.1-0.9 mM) under Standard Assay Conditions. The Activity Assay was Performed with 0.1 M and 0.3 M Urea as Described under Materials and Methods.

Furthermore, to investigate the mode of AHA binding in the active site, urease was suitably diluted with 0.1 M Tris-acetate buffer, pH 7.6 and incubated with 1 mM AHA for 25 min at 37°C in the absence of urea. The activity was determined with AHA inhibited and untreated urease and it was found that the activity was 17% after 25 min of incubation. The AHA inactivated urease (17% residual activity) was dialyzed extensively overnight against 0.1 M Tris-acetate buffer, pH 7.6 at 4°C. Next day the activity was determined and more than 74% activity was recovered. It clearly shows that AHA binding in the active site is reversible in nature.

From Table I, it is quite clear that the K_i values for the plant ureases are generally higher than that of bacterial sources. Clearly, the affinity of AHA for the active site of urease from different plant sources decreases in the following order: *Canavalia ensiformis* > *Cajanus cajan* > *Glycine max* > *Citrullus vulgaris*.

Table I : Comparison of I50 and K_i Values of Urease from some Major Sources.

Source	I50 (mM)	K_i (mM)
Citrullus vulgaris	0.70a	2.5a
Cajanus cajan	0.25b	0.0410b
Glycine max	0.90	0.0530
Canavalia ensiformis	--	0.0198c
Klebsiella aerogenes	--	0.0026d
Bacillus pasteurii	4.00e	--

Source: [a]Prakash and Upadhyay, 2004; [b]Srivastava et al., 2002; [c]Dixon et al., 1980; [d]Todd and Hausinger, 1989; [e]Benini et al., 2000.

Effect of pH on K_i

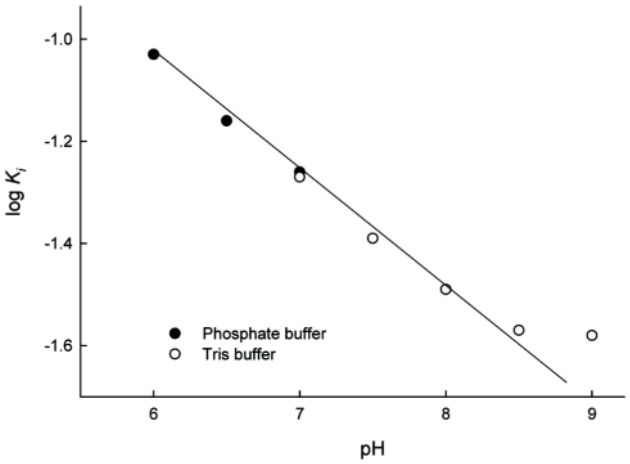


Figure 3 : Effect of pH on K_i Values for AHA Inhibition of Soybean Urease. The K_i Values were Determined by Dixon Plot Method. The Enzyme (0.87 µg/ml) was Incubated with Varying Concentrations of AHA in Phosphate Buffer (pH 6 to 7) and Tris-acetate Buffer (pH 7 to 9).

The soybean urease was further investigated to see whether AHA inhibition was pH dependent or not. The effect of pH on K_i was studied by determining the K_i values by Dixon method at different pH (6.0 – 9.0). Two

kind of buffers were used for this study viz., phosphate buffer (pH 6.0 to 7.0), and Tris-acetate buffer (pH 7.0 to 9.0). The log of K_i values were plotted against pH and a straight line with negative slope was obtained. From Figure 3, it is clear that the affinity of AHA for the active site decreases with decreasing value of pH (below the pK_a value of AHA i.e. 8.7). This behaviour is consistent with the deprotonated AHA acting as a nucleophile or the inhibitory species. The pH-dependence of K_i for soybean urease-AHA inhibition reveals a different mode of binding compared to microbial ureases (Todd and Hausinger 1989; Benini et al., 2000), and is similar to jack bean urease (Dixon et al., 1980) and pigeonpea urease (Srivastava et al., 2002).

The competitive inhibition of soybean urease was studied at 36°C in aqueous solution (pH 4.95) in the presence of polycarbonyl compounds (PCCs): oxalyldihydrazide (ODH), its polydisulfide {poly(DSODH)}, three cyclic beta-triketones (CTKs), and seven cyclic PCC species of differing structure. In these studies it was shown that the varying pH values (3.85 to 7.40) exerted a strong effect on the values of K_i of three CTKs and hydroxyurea. These pH dependences of log K_i were linear in all cases and displayed a break at pH 6.0–6.5. (Tarun et al., 2005). In other report the competitive inhibition of soybean urease by 15 triketone oximes was studied at 36°C in aqueous solution (pH 4.95). The K_i of urea hydrolysis varied in the range 2.7–248 µM depending on the oxime structure (Tarun et al., 2004a). The similar studies from soybean urease with 11 cyclic beta-triketones have established competitive type of inhibition at pH 7.4 and 36°C. The K_i showed a strong dependence on the structure of organic chelating agents (nickel atoms in urease) and varied from 58.4 to 847µM. Under similar conditions, the substrate analogue (hydroxyurea) acted as a weak urease inhibitor (K_i = 6.47 mM) (Tarun et al., 2004 b).

The AHA inhibits pigeonpea urease competitively and reversibly with a K_i of 0.041 mM at pH 7.3. The variations in pH exerted changes in the K_i values and a time-dependent inhibition was reported. AHA inhibition revealed biphasic kinetics (Srivastava et al., 2002). The report from watermelon urease has established that AHA acts as an uncompetitive inhibitor and has K_i 2.5 mM. Binding of AHA with the enzyme was shown reversible in nature (Prakash and Upadhyay, 2004).

Time-Dependent Inhibition Studies

The time-dependent inhibition studies at standard assay conditions revealed the biphasic kinetics (Figure 4). The enzyme lost its activity in two distinct phases. In the first phase the rate of loss in activity was quite fast, while in the second phase it was quite slow. The complete time course of inhibition was analyzed with equation (1). The amplitudes and rate constants were determined from the semi-log plots by the method described earlier (Kayastha and Gupta, 1987).

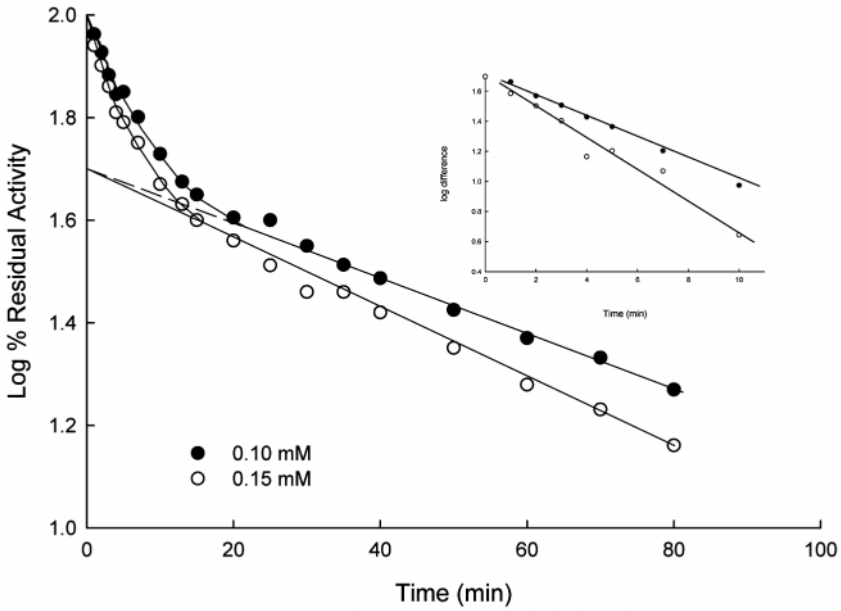


Figure 4 : Time-dependent Inhibition Studies of Soybean Urease with AHA. The Enzyme (2.28 µg/ml) and AHA (0.10 or 0.15 mM) were Incubated in 0.1 Tris-acetate Buffer, pH 7.6, and 37°C. Aliquots withdrawn at Specified Intervals were Assayed for Percent Residual Activity. The Inset Shows the Semi-log Plot for the Fast Phase.

The values of the amplitudes and rate constants at two inhibitor concentrations are shown in Table II. As evident from the results, half of the active sites of soybean urease were more reactive for AHA than the other half. These results are consistent with our earlier reports regarding half-site reactivity of active site of pigeonpea urease (Kayastha and Gupta, 1987; Kayastha and Das, 1998; Das et al., 2002; Srivastava et al., 2002).

Table II : First-order Rate Constants and Amplitude for the Inactivation/inhibition of Soybean Urease with 0.15 and 0.20 M AHA.

Inhibitor (mM)	*Fast phase*		*Slow phase*	
	A_{fast} *(%)*	k_{fast} *(min^{-1})*	A_{slow} *(%)*	k_{slow} *(min^{-1})*
0.15	49.9	0.1642±0.0013	50.1	0.0123±0.0012
0.20	49.9	0.2379±0.0017	50.1	0.0153±0.0010

Spectral Studies

The spectral studies showed a change in absorbance at the wavelength 414 nm (Figure 5). The AHA is a metal chelator, and has been shown to form a bidentate complex with Ni^{2+} ions and other ions in model system (Dixon et al., 1980) and also bring a change in absorption spectrum near 415±2 nm region (Stemmler, 1995). It alone does not have any absorbance in the specified range. It is suggested that the small absorbance change observed at 414 nm is probably due to the interaction of AHA with Ni ions present at the active site of soybean urease. Also it has been shown that the soybean urease has two Ni^{2+} ions per active site. This observation along with evidences from earlier studies demonstrates that AHA binds to Ni^{2+} ions in the active site of soybean urease. Similar direct spectrophotometric evidence of coordination of a hydroxamic acid inhibitor to active site Ni^{2+} ions have

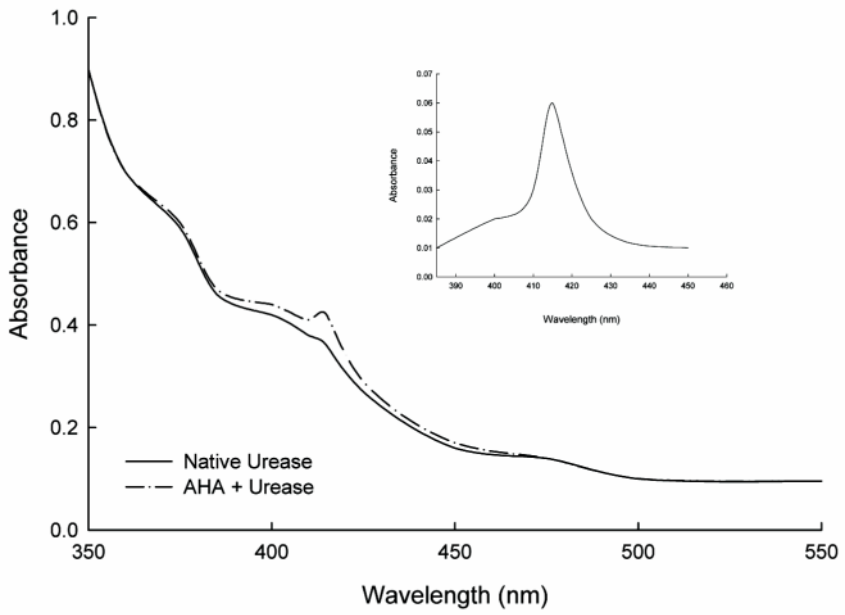
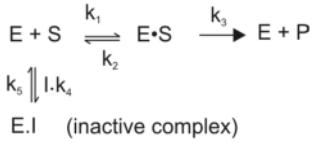


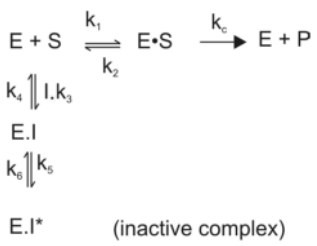
Figure 5 : The Effect of AHA on the Absorbance Spectrum of Soybean Urease. Soybean Urease (20 mg/ml) and AHA (12 mM) were Incubated for 25 min. and Spectra were Recorded on ATI-UNICAM UV-2 Spectrophotometer. Inset Shows the Difference Spectrum for the AHA Inhibited Soybean Urease.

been shown for jack bean urease (Dixon et al., 1980) and pigeonpea urease (Srivastava et al., 2002).

On the basis of our studies, we propose the same mechanism for soybean urease inhibition with AHA as proposed by Srivastava and Kayastha (2002) for pigeonpea urease inhibition of AHA. The E.I complex may involve the coordination of the AHA anion to the metallocentre, in which possibly a tetrahedral AHA-derived gem-diolate intermediate is formed, leading to the bridging of two Ni ions and forming a dead end complex as proposed for *B. pasteurii* urease (Benini et al., 2000).



Scheme 1



Scheme 2

In Scheme 1, the equilibrium constant k_5/k_4 was found to be the overall dissociation constant (K_i) for urease AHA inhibition, as compared to [$K_i^* = K_i(k_6 + k_5)/k_6$] in case of microbial ureases (Scheme 2) (Todd and Hausinger, 1989; Benini et al., 2000). Thus, the mechanisms of AHA binding to the bacterial and plant urease appear to be different (Scheme 1 *versus* Scheme 2). This distinction between the two kinetic mechanisms also arises from the ability to observe a limiting apparent binding rate (k_{app}) at higher inhibitor concentration with the *K. aerogenes* urease (Todd and Hausinger, 1989).

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