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# Reactivities of Oxalamidines (*N,N*-Oxalins)

MYEONG YUN CHAE AND GORDON A. HAMILTON

*Center for Biomolecular Structure and Function, Department of Chemistry, Pennsylvania State University, University Park, Pennsylvania 16802*

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In order to determine the reactivity characteristics of putative oxalamidine metabolites (monoamidines of oxalic acid or *N,N*-oxalins), a series of cyclic and acyclic derivatives were synthesized and their reactivities examined. The results indicate that around neutral pH at 37°C oxalamidines are stable to hydrolysis or attack by nucleophiles typical of those that would be encountered physiologically. Also, under such conditions metal ions do not appreciably catalyze their destruction. The results further indicate that oxalamidines are stable to acid conditions (at least to 1 M HCl) but that they are readily hydrolyzed under basic conditions to oxamate derivatives. Kinetic and product data for the base-catalyzed reaction can be analyzed in terms of a mechanism similar to that proposed earlier for the hydrolysis of other amidines. This involves reversible addition of hydroxide to the protonated amidine followed by rate determining loss of amine. 

## INTRODUCTION

Previous investigations in our laboratory (*1–10*) have led to the identification of various nucleophile–glyoxylate adducts as the probable physiological substrates for the mammalian peroxisomal enzymes, D-amino acid oxidase, D-asparate oxidase, and L-hydroxy acid oxidase. The products of all these reactions are oxalic acid derivatives. Since several of the products are metabolites (*11–13*) that appear to play an important role in the regulation of animal metabolism (*13–17*), we now refer generally to such oxalic acid derivatives as "oxalins." To distinguish among nitrogen, oxygen, and sulfur derivatives of oxalic acid the prefixes N, O, and S are used. Thus, monoamides of oxalic acid are referred to as *N*-oxalins, monoesters as *O*-oxalins and monothiol-esters as *S*-oxalins. Combinations of these can also occur, to indicate when the groups are attached to different carboxyl groups one of the prefixes is primed. Thus, diamides of oxalic acid are referred to as *N,N'*-oxalins, whereas monoamidines of oxalic acid are *N,N*-oxalins.

In earlier publications we have characterized the chemical reactivities of *S*-oxalins (*18*) (the products from the hydroxy acid oxidase-catalyzed reaction), *N,S*-oxalins (*19, 20*) (thioimidates, products from the amino acid oxidase-catalyzed reactions), and *O,S'*-oxalins (*21*). In the present article we report results that define some of the chemical reactivities of *N,N*-oxalins. It is unknown whether *N,N*-oxalins are animal metabolites but we recently reported (*10*) that one such compound (2-carboxy-4,5,6,7-tetrahydro-1,3-diazepine [cyclic(7)-oxalamidine], Scheme I) is formed *in vitro* by the D-amino acid oxidase-catalyzed oxidation of a glyoxylate



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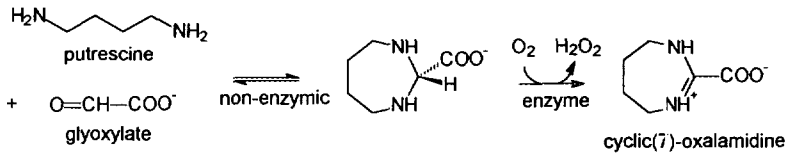



SCHEME I

adduct of putrescine (1,4-diaminobutane) under conditions similar to those *in vivo*. This compound was also found to be an effective inhibitor ($K_i$ = 20 $\mu$M) of D-amino acid oxidase. Since numerous investigations have suggested that the putrescine concentration is a marker for rapid cell proliferation (*22, 23*), the possibility was considered (*10*) that this *N,N*-oxalin might somehow be involved in the control of cell proliferation. Consequently, an investigation of the chemical stability and reactivity of *N,N*-oxalins seemed warranted.

The antibiotic kasugamycin (*24*) was the first reported example of an *N,N*-oxalin but since that time several others have been synthesized (*24–26*). However, very little is known about their chemical reactivities. There is some literature on amidine hydrolysis (*27–30*) but none of these studies has been concerned with oxalamidines (*N,N*-oxalins). In the present investigation several oxalamidines, both cyclic and acyclic, have been synthesized, their stability under physiological conditions determined, and their reactivity with nucleophiles, especially hydroxide ion, examined.

## EXPERIMENTAL

*Commercial materials.* All commercially available materials including organic solvents were of reagent grade quality or better and were used without further purification unless otherwise noted.

*Preparation of cyclic-oxalamidines.* Cyclic(7)-oxalamidine (2-carboxy-4,5,6,7-tetrahydro-1,3-diazepine) was prepared as described by Afeefy *et al.* (*10*). Cyclic(6)-oxalamidine (2-carboxy-1,4,5,6-tetrahydropyrimidine) was prepared by oxidation of 2-hydroxymethyl-1,4,5,6-tetrahydropyrimidine (*31*) with potassium permanganate using the same conditions as described for the preparation of cyclic(7)-oxalamidine (*10*). To isolate the cyclic(6)-oxalamidine, the white inorganic solids were first removed by filtration after adding ethanol to the reduced volume (approximately 4 ml) of aqueous solution. After evaporating the solvent under reduced pressure, a semisolid was obtained. This was purified by column chromatography on silica gel using 1:2 methanol:chloroform as the eluting solvent. The white solid (yield, 5%; mp, 166–170°C) gave only one spot on TLC ($R_f$ 0.4; 1:1 methanol:chloroform) using silica gel 60 $F_{254}$ precoated plates. $^1$H NMR (in $CD_3OD$) $\delta$: 1.95 (m, 2H), 3.45 (t, 4H); electron impact mass spectrum, *m/e* (relative intensity), 128 (0.8), 84 (100).

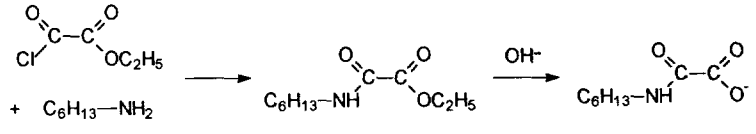
*Preparation of noncyclic oxalamidines.* These were prepared from potassium thionooxamate (*32*) and the corresponding amines by the procedure of Yoshimura


SCHEME II

*et al.* (*25*) (Scheme II). ***N*-Butyloxalamidine** (R = butyl): mp 190–191°C, dec. (lit. 192°C, dec.); $^1$H NMR (in $D_2O$) δ: 0.7 (t, 3H), 1.2 (m, 2H), 1.5 (m, 2H), 3.1 (t, 2H); positive fast atom bombardment ionization mass spectrum, *m/z* 145 (MW = 144 amu). ***N*-Hexyloxalamidine** (R = hexyl): mp 163–164°C, dec. (lit. 164–165°C, dec.); $^1$H NMR (in $D_2O$) δ: 0.7 (t, 3H), 1.2 (m, 4H), 1.4 (m, 2H), 3.1 (t, 2H); positive fast atom bombardment ionization mass spectrum *m/z* 173 (MW = 172 amu). ***N*-Ethyloxalamidine** (R = ethyl): mp 170–171°C, dec. (lit. 177–178°C, dec.); $^1$H NMR (in $D_2O$) δ: 1.1 (t, 3H), 3.2 (q, 2H); positive fast atom bombardment ionization mass spectrum *m/z* 117 (MW = 116 amu). **Oxalamidine** (R = H): the brown solid obtained could not be purified because of very low solubility in all solvents examined; positive fast atom bombardment ionization mass spectrum *m/z* 89 (MW = 88 amu).

*Preparation of N-(4-aminobutyl)-oxamate.* This was prepared by the base-catalyzed hydrolysis of cyclic(7)-oxalamidine. After dissolving the oxalamidine (284 mg, 2 mmol) in 30 ml water, the solution was placed in a 37°C water bath and 1.0 N NaOH was added intermittently to keep the pH at approximately 12.5. The reaction was monitored both by TLC analysis and spectrophotometrically. When the reaction is completed (about 1.5 h), the pH of the solution was readjusted to 7.5 with 1.0 N HCl. The white solid obtained after evaporation of the water was leached with methanol and the methanol evaporated. TLC and NMR indicated the presence of only one organic compound in the material so obtained; thus, it was not further purified. However, the material contained a considerable amount of inorganic impurity (probably NaCl) since the uv absorbance of this material was reduced compared to that obtained following complete base-catalyzed hydrolysis of a known concentration of cyclic(7)-oxalamidine. NMR $^1$H (in $D_2O$) δ: 1.6 (m, 4H), 2.9 (t, 2H), 3.2 (t, 2H); $^{13}$C (in $D_2O$) δ: 24.2, 25.4, 38.7, 39.2, 165.2, 166.2.

*Preparation of N-hexyloxamate.* This was prepared as outlined in Scheme III. Hexylamine (10.12 g, 0.1 mol) was added dropwise over 20 min to a solution of ethyl oxalyl chloride (6.83 g, 0.05 mol) in 400 ml anhydrous ether in a 1-liter round-bottomed flask packed in ice. The white solid which formed (presumably hexylamine · HCl) was removed by filtration, and the filtrate was dried with $MgSO_4$. After removing the solvent, the oily residue was distilled under reduced pressure. The collected colorless liquid (presumably the ethyl ester of *N*-hexyloxamate) had



SCHEME III

the following characteristics: bp, 102–105°C (0.2 mm Hg); $^1$H NMR (in $CDCl_3$) δ: 7.1 (broad s, NH, 1H), 3.3 (q, 2H), 1.5 (m, 2H), 1.2–1.4 (m, 11H), 0.85 (t, 3H); chemical ionization mass spectrum, 202 *m/z* (MW = 201 amu). To 0.5 g (2.5 mmol) of this ester with 4 ml of water (2 phases) was added 1.0 N NaOH continuously with vigorous stirring to keep the pH of the solution at approximately 9–11. When the pH no longer changed (approximately 4 h), the solution was extracted with 10 ml chloroform and the extract discarded. The pH of the aqueous solution was adjusted to 10, and the solvent removed by rotary evaporation The white solid that was obtained had the following characteristics: $^1$H NMR (in $D_2O$) δ: 3.1 (t, 2H), 1.4 (m, 2H), 1.1 (m, 6H), 0.7 (t, 3H); $^{13}$C NMR (in $D_2O$) δ: 13.7, 22.2, 26.1, 28.6, 31.1, 39.7, 165.2, 166.3; negative fast atom bombardment ionization mass spectrum *m/z* 194 (MW = 195 amu).

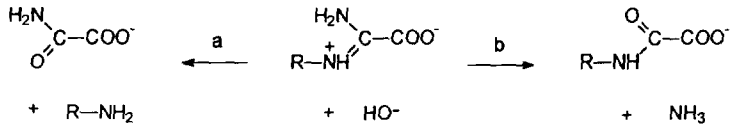
*Products from the hydrolysis of N-hexyloxalamidine.* *N*-Hexyloxalamidine (34 mg, 0.2 mmol) in 4 ml of 0.05 N NaOH in a closed vial was incubated at 37°C until the uv absorption at 244 nm indicated the reaction was complete (3 h). After adjusting the pH to 2.0 with 1.0 N HCl, the solvent water was evaporated under reduced pressure and the resulting white solid examined by TLC and $^1$H NMR in $D_2O$.

*Kinetic experiments.* Each of the oxalamidines examined has an absorbance maximum (or shoulder) at 236 to 244 nm (extinction coefficient 3000 ± 600 $M^{-1}$ $cm^{-1}$ at pH 12.6, 1.0 M KCl) while the hydrolysis products have only weak end absorption at these wavelengths. For the cyclic oxalamidines the largest change occurs at 244 nm, for the *N*-alkyloxalamidines at 240 nm, and for the unsubstituted compound at 236 nm, so the hydrolysis of each respective compound was followed by monitoring the spectral change at those wavelengths for 10–15 half-times. All reactions were performed under pseudo-first-order conditions and good first-order kinetics were observed for at least three half-times. The observed first-order rate constants ($k_{obs}$) were obtained and further analyses of the data were performed by linear regression analyses using Statgraphics.

All reactions were run at 37.0°C. For reactions involving the cyclic and alkyl oxalamidines the solutions contained initially 0.25 to 0.30 mM oxalamidine, 1.0 M KCl, and 4.6 to 135 mM hydroxide ion, the latter obtained by adding aliquots of a carbonate-free aqueous NaOH solution. The hydroxide ion concentration of each reaction solution was determined by titration of a degassed aliquot with a standard HCl solution just before running the reaction. With oxalamidine as reactant, its concentration was not known, and, because it is so much more reactive, lower concentrations of hydroxide ion (0.7 to 20 mM) were used. At the lowest concentrations pseudo-first-order kinetics would not strictly apply, but these data would affect only the calculation of the $K_a$ for oxalamidine. For this reason the $K_a$ obtained for this compound is less accurate than those obtained for the other compounds.

## RESULTS

*General observations on reactivity of oxalamidines.* By monitoring any absorption changes at approximately 240 nm where the oxalamidines absorb, it was found in preliminary experiments that they are stable in neutral and acidic (to 1 *M* HCl)



SCHEME IV

solutions but a reaction occurs under basic conditions (pH $\geq$ 10). As described in the next section, this reaction has been studied in some detail. In other preliminary experiments to characterize oxalamidine reactivity, it was shown that at pH 7.4 (100 m*M* phosphate buffer) and 37°C there is no detectable reaction of either cyclic(7)-oxalamidine or *N*-butyloxalamidine with 20 m*M* concentrations of hydroxylamine and hydrogen peroxide, nor was any reaction observed with 5 m*M* cysteine or 2.5 m*M* cysteamine under these conditions. Similarly, metal ions do not catalyze the hydrolysis reaction under physiological conditions. Thus, at pH 7.5 (10 m*M* phosphate buffer, 1.0 *M* KCl) at 37°C, there was no detectable change in absorbance at 244 nm when cyclic(7)-oxalamidine (0.3 m*M*) was incubated with 0.5 m*M* concentrations of $Mg^{2+}$, $Ni^{2+}$, $Pb^{2+}$, or $Cu^{2+}$. The same negative result was obtained when the incubations were carried out at pH 8.5 in 50 m*M* carbonate buffer. At higher pH it is difficult to obtain quantitative data because the metal ions precipitate, but qualitatively there did not appear to be any catalysis of the hydrolysis reaction when such metal ions were present.

*Products from the base-catalyzed hydrolysis of oxalamidines.* As indicated previously, the absorbances at about 240 nm of all the *N, N*-oxalins decrease with time in basic solution due to hydrolysis. The product from the hydrolysis of cyclic(7)-oxalamidine was shown unequivocally to be *N*-(4-aminobutyl)-oxamate as indicated by its $^1H$ NMR and $^{13}C$ NMR spectra (see Experimental). Although a detailed product analysis was not performed for all the other *N, N*-oxalins, there can be little doubt from the spectral changes observed that these *N-N*-oxalins also hydrolyze to give oxamate derivatives.

Since the acyclic *N*-alkyloxalamidines could hydrolyze to give either oxamate and the alkyl amine (pathway, a, Scheme IV) or *N*-alkyloxamate and ammonia (pathway b), some experiments were performed to determine which occurs or is favored. In a preliminary study, the hydrolysis products of both *N*-butyloxalamidine and *N*-hexyloxalamidine (in each case 10 m*M* oxalamidine was reacted with 39 m*M* NaOH in 1.0 *M* KCl at 37°C for 1.5 h) were analyzed by TLC using the solvent system butanol/acetic acid/$H_2O$ (60/15/25, v/v/v) and a ninhydrin spray. It was observed that very strong ninhydrin-active spots appeared at $R_f$ = 0.3 following the reaction of *N*-butyloxalamidine and at $R_f$ = 0.45 following the reaction of *N*-hexyloxalamidine. These $R_f$ values are the same as those for butylamine and hexylamine respectively. Thus, at least part of the reaction must go by pathway a, Scheme IV.

To obtain a more quantitative estimate of the relative importance of pathways

TABLE 1
Kinetic Data for the Base-Catalyzed Hydrolysis of Oxalamidines at 37°C[a]

| Reactant | $Q$ ($M^{-1}$ s$^{-1}$) | $K_a$ ($M \times 10^{12}$) | p$K_a$ |
|---|---|---|---|
| Cyclic(7)-oxalamidine | 0.045 | | >13 |
| Cyclic(6)-oxalamidine | 0.028 | 0.079 | 13.1 |
| *N*-ethyloxalamidine | 0.128 | 1.65 | 11.8 |
| *N*-butyloxalamidine | 0.122 | 1.66 | 11.8 |
| *N*-hexyloxalamidine | 0.110 | 1.51 | 11.8 |
| Oxalamidine | 1.43 | 5.9 | 11.2 |

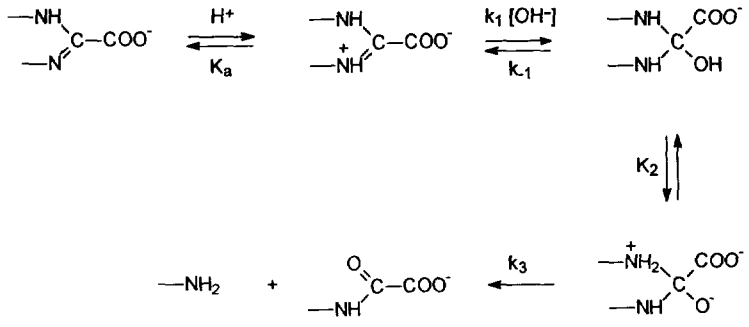
[a] See text for reaction conditions and a definition of each of the constants.

a and b in the hydrolysis of the acyclic *N*-alkyloxalamidines, the acidified hydrolysis products obtained from *N*-hexyloxalamidine were examined by $^1$H NMR. The $^1$H NMR peaks of the hydrolysis products were virtually the same as those of hexylamine · HCl [in $D_2O$: δ 2.85 (t, 2H) 1.5 (m, 2H), 1.2 (m, 6H), 0.7 (t, 3H)] except for a very small triplet peak at 3.1 ppm which was at the same position as a triplet peak found in the spectrum of *N*-hexyloxamate. The results indicate that pathways a and b both occur, but pathway b accounts for only 5–10% of the total reaction.

*Kinetic data for the base-catalyzed hydrolysis reactions.* When the observed first-order rate constant ($k_{obs}$) for the base-catalyzed hydrolysis of cyclic(7)-oxalamidine is plotted versus the hydroxide concentration, a straight line that goes through the origin is obtained for the range of hydroxide ion concentrations utilized (20 to 98 m*M*). From the data one can thus calculate a second-order rate constant for the reaction and it is 0.045 $M^{-1}$ $s^{-1}$. For all the other oxalamidines studied, however, similar plots do not give straight lines; in such cases the rate tends toward a constant maximum rate at high hydroxide ion concentrations. For reasons that will be elaborated in the Discussion, it is expected that $k_{obs}$ should be related to the hydrogen ion concentration as shown in Eq. [1] where $Q$ is a constant, $K_w$ is the ionization constant for water, and $K_a$ is the acid ionization constant for the oxalamidinium species. The reciprocal relation (Eq. [2]) predicts that a plot of $1/k_{obs}$ versus [$H^+$] should be a straight line. This was found to be the case for all the oxalamidines. Knowing $K_w$ [$pK_w$ was taken as 13.97 (*33*)] one can obtain from the slopes and intercepts of these lines values for $Q$ and $K_a$ for each of the compounds. These values, as well as values for each p$K_a$ are given in Table 1:

$$k_{obs} = \frac{Qk_w}{[H^+] + K_a} \quad [1]$$

$$\frac{1}{k_{obs}} = \frac{[H^+]}{QK_w} + \frac{K_a}{QK_w}. \quad [2]$$



SCHEME V

## DISCUSSION

The results reported here indicate that around neutral pH at 37°C oxalamidines are stable to hydrolysis or attack by nucleophiles typical of those that would be encountered physiologically. Also, under such conditions metal ions do not appreciably catalyze their destruction. Thus, if oxalamidines are formed *in vivo* they should be detectable unless their degradation is rapidly catalyzed by some enzyme.

The current results further indicate that oxalamidines are stable to acid (at least to 1 *M* HCl) but that they are readily hydrolyzed in base to oxamate derivatives. From earlier work on the hydrolysis of amidines (*27–30*), one suspects that the base-catalyzed hydrolysis of the oxalamidines will proceed as outlined in Scheme V. Assuming the mechanism of Scheme V, one can derive that the pseudo-first-order rate constant ($k_{obs}$) should be as given in Eq. [3] where $K_a$ is the acid ionization constant for the oxalamidinium ion, and $K_2$ is defined as the concentration ratio of the zwitterion to the neutral form of the tetrahedral intermediate (ignoring the charge on the carboxylate which would be present under all conditions). As can be seen, Eq. [3] has the same form as Eq. [1] where $Q$ is just equal to the expression given in Eq. [4]. Consequently, the detailed kinetic results are consistent with the mechanism of Scheme V for the base-catalyzed hydrolysis reaction:

$$k_{obs} = \frac{k_1 k_3 K_2 K_w}{([H^+] + K_a)(k_{-1} + k_3 K_2)} \qquad [3]$$

$$Q = \frac{k_1 k_3 K_2}{k_{-1} + k_3 K_2}. \qquad [4]$$

For none of the oxalamidines investigated was any evidence obtained, either spectrally or kinetically (good first order kinetics were observed), for any appreciable buildup of a tetrahedral intermediate as was observed during the hydrolysis of a diphenylformamidinium compound (*29*). However, even though the intermediate is present only in very small amounts during the reaction, there are still two mecha-

nistic extremes possible for an overall mechanism as in Scheme V: (i) if $k_{-1}$ is small relative to $k_3K_2$, the attack of hydroxide on the oxalamidinium ion would then be rate determining, and (ii) if $k_3K_2$ is small relative to $k_{-1}$, the decomposition of the tetrahedral intermediate to the products oxamate and amine would then be rate determining and the hydroxide addition step would be a rapid equilibrium. If (i) applies the experimentally derived constant $Q$ would just be equal to the second-order rate constant $k_1$ for attack of hydroxide ion on the protonated oxalamidine, whereas if (ii) applies $Q$ would be equal to $k_3K_1K_2$ (where $K_1 = k_1/k_{-1}$).

Although the current results cannot distinguish which of the mechanistic extremes [(i) or (ii), or even a combination of the two] is the correct one for the base-catalyzed oxalamidine hydrolysis, it seems probable, by analogy to earlier results with other amidines (*27–30*), that mechanistic extreme (ii) applies. As in the other cases, it is expected that loss of hydroxide from the initially formed carbinolamine (Scheme V) would occur much more rapidly than its breakdown to amine and oxamate, especially since the oxalamidinium ions are so stable (have high $pK_a$ values, see Table 1). If such is the case, then $Q$ is a product of three constants and it becomes difficult to come to firm conclusions concerning the reasons for changes in $Q$ as a function of oxalamidine structure. Nevertheless some suggestions can be offered. Probably the constant in $Q$ that depends most on oxalamidine structure is $K_1$, the equilibrium constant for hydroxide addition to the oxalamidinium ions. It is expected that $K_1$ would vary inversely with the stability of the oxalamidinium ions and that may be one of the main reasons that $Q$ decreases as $pK_a$ increases (Table 1). For oxalamidine itself, a favorable steric factor may also be operating to increase $Q$ even more relative to the substituted derivatives. With less steric crowding in the carbinolamine formed from oxalamidine, $K_1$ should be increased, and if so, would cause a further elevation of $Q$ as observed.

The observation that unsymmetrical alkyloxalamidines hydrolyze mainly to alkylamine and oxamate (pathway a, Scheme IV) rather than ammonia and alkyloxamate (pathway b) is easily understood on the basis of the mechanism in Scheme V. The particular products formed will be largely controlled by the $K_2$ step regardless whether mechanistic extreme (i) or (ii) is the correct one. Since alkylamines are more basic than ammonia (the $pK_a$ for ammonium ion is 9.2, whereas it is about 10 for alkylammonium ions), protonation on the alkyl substituted nitrogen of the carbinolamine will thus be favored in the $K_2$ step. Subsequent cleavage of this intermediate would then be expected to lead to more of the products being alkylamine and oxamate as observed.

There are very few data in the literature on the base-catalyzed hydrolysis of simple amidines that can be profitably compared with the results found here for oxalamidines. The data obtained by Burdick *et al.* (*30*) for the hydrolysis of cyclic and acyclic phenyl-substituted formamidines is perhaps the best available for such a comparison, even though the presence of the phenyl group complicates the comparison (for example, it lowers the $pK_a$ values of the conjugate acids to approximately 9 or 10). In any event, the $Q$ values obtained by Burdick *et al.* for the base-catalyzed hydrolysis of the formamidines are two to three orders of magnitude greater than those found here for the oxalamidines. This difference for hydrogen substituted derivatives (formyl) relative to carboxylate substituted ones (oxalyl) is

fairly typical for reactions that involve the addition of a nucleophile in a preequilibrium step (*6*). By this comparison then the reactivity of oxalamidines toward base, found in the current work, is about that expected.

The lack of observable metal on catalysis of oxalamidine hydrolysis merits some comment, especially since Angelici and his co-workers (*34, 35*) reported that metal ions do catalyze the hydrolyses of both esters and thiolesters of oxalate. They found that the rate of hydroxide attack on both types of compounds is up to $10^5$ times greater when the ester or thiolester is coordinated to a metal ion than when free in solution. Presumably by coordinating to the reacting carbonyl group the metal ion polarizes the bond, thus making it more suspectible to attack by the hydroxide nucleophile in the rate determining step of these reactions. It is expected that oxalamidines should also form bidentate complexes with metal ions and that the metal ion would polarize the carbon–nitrogen double bond. However, the metal ion would probably not be as effective in polarizing the bond as direct protonation of the nitrogen, a step that occurs in the non-metal-catalyzed reaction (Scheme V). In any event, since the attack of hydroxide is not the slow step in the amidine reaction, even if the metal ion accelerated this step, that alone would not affect the rate of the overall hydrolysis. It is difficult to predict what effect coordination of a metal ion would have on the $K_2$ and $k_3$ steps, but it would probably be minor. Consequently, in retrospect it is not too surprising that metal ions have no detectable effect on the base-catalyzed hydrolysis of the oxalamidines.

## ACKNOWLEDGMENTS

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