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Direct Observation of Acyl Nitroso Compounds in Aqueous Solution and the

Kinetics of their Reactions with Amines, Thiols and Hydroxamic Acids

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Abstract

Acyl nitroso compounds or nitrosocarhonyls (RC(O)N=O) are reactive short-lived electrophiles, and their hydrolysis and reactions with nucleophiles produce HNO. Previously, direct detection of acyl nitroso species in non-aqueous media has been provided by time-resolved infrared spectroscopy demonstrating that its half-life is about 1 ms. In the present study hydroxamic acids (RC(O)NHOH) are oxidized electrochemically in buffered aqueous solutions (pH 5.9 – 10.2) yielding transient species characterized by their maximal absorption at 314 – 330 nm. These transient species decompose *via* a first-order reaction yielding mainly HNO and the respective carboxylic acid, and therefore are ascribed to RC(O)N=O. The sufficiently long half-life of RC(O)N=O in aqueous solution allows for the first time the study of the kinetics of its reactions with various nucleophiles demonstrating that the nucleophilic reactivity follows the order thiolate > hydroxamate > amine. Metal chelats of CH₃C(O)NHOH catalyze the hydrolysis of CH₃C(O)N=O at the efficacy order of Cu^{II} > Zn^{II} > Ni^{II} > Co^{II} where only Cu^{II} catalyzes the hydrolysis also in the absence of the hydroxamate. Finally, oxidation of hydroxamic acids generates HNO and the rate of this process is determined by the half-life of the respective acyl nitroso compound.

Introduction

Acyl nitroso compounds or nitrosocarhonyls (RC(O)N=O) have been proposed as transient intermediates during periodate oxidation of hydroxamic acid (RC(O)NHOH, HX),¹⁻⁴ by thermal fragmentation of Diels-Alder adducts⁵⁻⁷ and by photolysis of various precursors including 9,10-dimethylanthracene adducts, nitrodiazo compounds, and 1,2,4-oxadiazole-4-oxides⁸. Acyl nitroso compounds are reactive electrophiles, and their reactions with nucleophiles (NuH) produce nitroxyl (HNO)^{4,8-11}, which undergoes dimerization followed by dehydration yielding nitrous oxide (N₂O)¹² and/or reacts with various substrates such as thiols¹³.

$$RC(O)N=O + NuH \rightarrow RC(O)Nu + HNO$$
(1)

No direct detection of RC(O)N=O in aqueous solution have been previously reported. In most cases where the intermediacy of RC(O)N=O has been invoked, its generation has been indirectly confirmed by trapping it with conjugated 1,3-dienes that produces stable cycloadducts⁴ or by detection of $N_2O^{4-6,8,11,14,15}$. Spectroscopic evidence has been reported for HC(O)N=O in the gas phase,¹⁶ and various RC(O)N=O by time-resolved infrared spectroscopy where the lifetime has been estimated to be about 1 ms in non-aqueous solutions^{8,17}.

We have previously demonstrated that in aqueous solutions one-electron oxidation of acetohydroxamic acid and suberoylanilide hydroxamic acid (SAHA) by radiolytically-borne radicals such as 'OH, 'N₃ and Br₂⁻⁻ forms the respective transient nitroxide radicals (RC(O)NHO'/RC(O)NO'⁻⁻), which decompose bimolecularly yielding transient species with a maximum absorption at 314 nm.^{10,11} We have detected these transient species also upon oxidation of RC(O)NO'⁻⁻ by 'NO₂ and during the enzymatic oxidation of HX by horseradish peroxidase/H₂O₂.¹⁸ The decomposition of these transient species yields N₂O, their half-life decreases upon increasing the pH and/or [HX], and therefore they have been assigned to RC(O)N=O.^{10,11,18} Yet, their half-life in aqueous media is orders of magnitude longer than that

reported in non-aqueous solutions.^{8,17} In the present study various HXs are oxidized electrochemically yielding transient species with a maximum absorption at 314 - 330 nm that decompose to yield N₂O and the respective carboxylic acid. These transient species are attributable to acyl nitroso compounds, and their sufficiently long half-life allows for the first time the study of their kinetics with various nucleophiles in aqueous solutions.

Experimental Section

Materials

Water for preparation of the solutions was purified using a Milli-Q purification system. All chemicals purchased from Sigma-Aldrich were of the highest available grade and were used as received: acetohydroxamic acid (aceto-HX), suberohydroxamic acid (subero-HX), benzohydroxamic acid (benzo-HX), glycine hydroxamic acid (gly-HX), α -aminobutyric hydroxamic acid (AABA-HX), L-lysine hydroxamate hydrochloride (lys-HX), deferoxamine mesylate salt (DFO), acetic glacial acid (99.8%), benzoic acid, dimethylamine hydrochloride, ethylamine (70% solution), morpholine, L-cysteine, glutathione (GSH), N-acetylcysteine (NAC), glycine, (3-(N-morpholino) propanesulfonic acid (MOPS), 2-(N-Morpholino)ethanesulfonic acid, 4morpholineethanesulfonic acid (MES), [tris(hydroxymethyl)methylamino]propanesulfonic acid (TAPS) and Cu(ClO₄) 6H₂O. FeCl₃ 6H₂O, NiSO₄ H₂O and ZnSO₄ 7H₂O were obtained from British Drug Houses (BDH). Suberoylanilide hydroxamic acid (SAHA) was purchased from LC Laboratories. Stock solutions of SAHA and subero-HX were prepared in dimethyl sulfoxide (DMSO). Stock solutions of amines and thiols were prepared in the same buffer and pH studied. Most of the experiments were carried out in the presence of organic buffers to avoid the interference of phosphate ions with the binding of metal ions to the hydroxamic acids. Nitrite was assayed using the Griess reagent. The absorption at 540 nm was read 15 min after mixing the reagent with the sample. Calibration curves were prepared using known concentrations of nitrite.

Bulk electrolysis

The applied voltage was controlled using the EmStat-PalmSens USB powered potentiostat electrochemical interface. Home-made electro-chemical cell was used for bulk electrolysis. The cell consisted of a working electrode of graphite grains packed inside a porous Vycor glass tube (5 mm I.D.), through which the solutions were pumped. An outer glass cylinder, with separate electrolyte (10 mM phosphate buffer, pH 7.0) contained the platinum auxiliary electrode and Ag/AgCl (3.5 M) as a reference electrode (205 mV vs. NHE). Solutions were pumped at 165 μ L s⁻¹ flow rate into a 1 cm optical cuvette positioned within a HP 8453 diode array spectrophotometer and/or into a glass vial sealed with a rubber septum for N₂O determination by gas chromatography. All experiments were carried out at room temperature.

Gas chromatography (GC)

GC was used to determine the yield of N₂O, which is the product of HNO dimerization followed by dehydration. Anoxic solutions of HXs (6.5 - 7.5 mL) were pumped through the electrolysis cell into anoxic glass vials (10.7 mL). A gas aliquot of the reaction headspace (2 mL) was taken and 1 mL was injected at room pressure and temperature onto a 5890 Hewlett-Packard gas chromatograph equipped with a thermal conductivity detector, a 10 ft - 1/8 inch Porapak Q column at an operating oven temperature of 70 °C (injector and detector 150 °C) and a flow rate of 20 mL/min (He, carrier gas). The yields of N₂O were calculated on the basis of a standard curve prepared by injecting 1 mL of known amounts of N₂O gas (Maxima, Israel).

High Performance Liquid Chromatography (HPLC)

HPLC was used to determine the yields of benzoic acid and acetic acid as end-products of the electro-oxidation of benzo-HX and acetoHX, respectively. The HPLC system (Knauer, Germany) was equipped with an Azura P6.1L pump and an Azura UVD 2.1L detector. Samples (20μ L) were injected onto a YMC C18 column (150 x 4.6 mm I.D, 3 μ m). Chromatographic separations were performed at room temperature using an isocratic mobile phase consisting of a 50:50 solution of acetonitrile:phosphate buffer pH 2.6 in the case of benzo-HX and benzoic acid, and a 10:90 solution

of the same solvents for samples containing aceto-HX and acetic acid. The flow rate was 1.0 ml min⁻¹. In order to reduce solvent noise in the chromatogram, samples were first evaporated in a rotor-evaporator and then dissolved in the same solvent of the mobile phase. The analyte concentration remained constant throughout the process. Solutions of benxo-HX and benzoic acid were used as standards. Detection was at 215 nm. Peak identification was based on retention times compared with those of selected standards. Quantitation was based on calibration curves obtained by plotting peak area vs. concentration (0 - 500 μ M).

Aceto-HX and acetic acid were analyzed using the standard addition method, and detected at 195 nm. Acetic acid and aceto-HX at different concentrations ranging between 30% and 250% of the analyte concentration were added to a known volume of analyte solution. The addition volume did not exceed 1% of the total sample volume.

Results and Discussion

The structures of HXs used in this study and their $pK_a(-NHOH)$ are given in Fig. 1.





Electro-oxidation of HXs in 0.1 M KCl aqueous media buffered at pH 5.9 – 10.2 generates relatively long-lived transient species having a maximum absorption at 314 – 330 nm. Since there is

a limited data on $E_{1/2}$ and onset potential values of aceto-HX in aqueous media,²⁶⁻²⁸ the applied voltage was varied until a maximal absorption was achieved. Figure 2 shows the spectra of the transient species derived from electro-oxidation of aceto-HX, benzo-HX, SAHA and subero-HX at pH 7.3. These transient species decay *via* a first order reaction (Figure 2, inset, $\tau_{1/2} > 1.5$ min) implying that any depletion during the measurements (*ca.* 10 s) is negligible. Same k_d and ε_{314} values were obtained under anoxic conditions.



Figure 2. Spectra of transient species formed upon electro-oxidation of HXs in aerated solutions containing 0.1 M KCl and 20 mM MOPS buffer at pH 7.3. Inset: first order kinetics of the decay monitored at λ_{max} . (A) 500 μ M acet-HX, $k_d = 0.19 \pm 0.01 \text{ min}^{-1}$; (B) 150 μ M benzo-HX, $k_d = 0.46 \pm 0.02 \text{ min}^{-1}$; (C) 230 μ M SAHA, $k_d = 0.14 \pm 0.01 \text{ min}^{-1}$; (D) 95 μ M subero-HX, $k_d = 0.21 \pm 0.01 \text{ min}^{-1}$.

The ε_{max} -values have been calculated from the dependence of A_{max} on [HX]_o at the concentrations range where A_{max} increases proportionally with [HX]_o (Figure 3). These calculations are based on the assumption that no appreciable depletion of the transient species thus formed takes place until measurement, and that one molecule of HX yields one molecule of a transient species.



Figure 3. Dependence of A_{max} of the transient species derived from electro-oxidation of HX on [HX]_o in 0.1 M KCl and 20 mM MOPS buffer at pH 7.3.

In the case of alkylamino hydroxamic acids, which are less basic than alkyl hydroxamic acids (Figure 1), the half-life of the transient species are relatively short (Figure 4, inset) and highly dependent on $[HX]_0$. In these cases a lower limit for ε_{max} is set at relatively low $[HX]_0$. The ε_{max} -values are summarized in Table 1.



Figure 4. Spectra of transient species formed by electro-oxidation of 15 μ M AABA-HX (A) or gly-HX (B) in 0.1 M KCl and 20 mM MOPS buffer at pH 7.3. Inset: first-order decay kinetics of the transient species monitored at 325 nm, $k_d = 1.56 \pm 0.08 \text{ min}^{-1}$ (A) and at 317 nm, $k_d = 0.96 \pm 0.05 \text{ min}^{-1}$ (B).

HX	λ_{max} (nm)	$\epsilon (M^{-1} cm^{-1})$
subero-HX	314	3050 ± 150
aceto-HX	314	2150 ± 100
benzo-HX	330	1650 ± 100
SAHA	314	1450 ± 100
gly-HX	317	\geq 2200
AABA-HX	325	\geq 2200

Table 1. ε_{max} -values of the transient species formed by electro-oxidation of HX.^{*}

*Assuming that electro-oxidation of HX is complete and that each molecule of HX yields one molecule of a transient species. All solution contained 0.1 M KCl and 20 mM MOPS buffer at pH 7.3.

The decomposition of the transient species produces N₂O formed *via* HNO dimerization followed by dehydration. Accumulation of N₂O was determined following electro-oxidation of HX in 0.1 M KCl and 20 mM MOPS buffer at pH 7.3 under anoxia to avoid HNO reaction with O_2^{29} . Anoxic solutions (6.5 – 7.5 mL) were pumped through the electrolysis cell into an anoxic glass vial and 1 mL into an optical cuvette for the determination of the oxidation yield (A_{max}). A gas aliquot of the reaction headspace was taken either immediately following the electro-oxidation or 40 min later. Accumulation of N₂O immediately following the electro-oxidation was below the detection limit whereas that measured when the decay of the transient species was completed depends on the initial concentration of the transient species (Table 2).

HX	A_{\max}	[transient] _o , µM	[N ₂ O], µM	HNO yield, %
aceto-HX	0.21 ^{<i>a</i>}	98	35	71
	0.37	172	63	73
	1.08	500	182	72
	1.61	750	260	69
benzo-HX	0.23	139	61	88
	0.29	176	87	99
	0.33	200	91	91
SAHA	0.20	0.20	30	44
	0.29	0.29	45	45

Table 2. N₂O yields formed upon electro-oxidation of HX at pH 7.3 (20 mM MOPS buffer).

The experimental error is $\pm 10\%$.

a - 10 mM phosphate buffer.

Accumulation of nitrite at the end of the electro-oxidation process was the same as that measured when the decay of the transient species was completed. In the case of SAHA (where N_2O yield is relatively low) and in the case of benzo-HX (where N_2O yield is maximal), the accumulation of nitrite was less than 4% implying that there is no clear reciprocal ratio between the yields of HNO and nitrite.

HPLC measurements were performed to identify and quantify acetic acid and benzoic acid as endproducts of the electro-oxidation of aceto-HX and benzo-HX, respectively, at pH 7.3. The results demonstrate that each molecule of the transient species decomposes to yield one molecule of the respective carboxylic acid (Table 3). The residual concentration of benzo-HX was negligible, and it was significant (114 μ M) in the case where oxidation of 1 mM aceto-HX was incomplete resulted in 837 μ M transient species.

HX	A_{\max}	[transient] _o , µM	[RC(O)OH], µM	RC(O)OH yield, %
aceto-HX	1.08	500	479	96
	1.61	750	773	103
	1.80	837	818	98
benzo-HX	0.073	44	41	93
	0.125	76	85	112
	0.147	89	83	93
	0.21	127	115	91
	0.256	155	160	103
	0.27	164	146	89
	0.43	260	262	101

Table 3. Carboxylic acid yields formed upon electro-oxidation of HX at pH 7.3.

The experimental error is $\pm 10\%$.

The transient species is assigned to RC(O)N=O since upon hydrolysis it generates HNO and the respective carboxylic acid. In addition, the rate of its decomposition increases as the pH increases and as shown below it reacts also with various nucleophiles. RC(O)N=O can be formed *via* two consecutive one-electron oxidation processes (reactions 2 and 3) or a single one-electron oxidation process followed by dismutation of the nitroxide radical (reactions 2 and 4).

$$RC(O)NHOH \rightarrow RC(O)NHO' + H^+ + e^-$$
(2)

$$RC(O)NHO' \rightarrow RC(O)N=O + H^{+} + e^{-}$$
(3)

$$2 \text{ RC(O)NHO} \rightarrow \text{RC(O)NHOH} + \text{RC(O)N=O}$$
(4)

Both processes lead to the oxidation of RC(O)NHOH to RC(O)N=O, which decomposes *via* hydrolysis yielding the respective carboxylic acid and HNO (reaction 5) that subsequently forms N_2O (equation 6).

$$RC(O)N=O + H_2O \rightarrow HNO + RC(O)O^- + H^+$$
(5)

$$2 \text{ HNO} \rightarrow \text{HONNOH} \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O} \tag{6}$$

HNO yields lower than 100% imply that while one-electron oxidation takes place, RC(O)NHO[•] might also decompose *via* dimerization yielding products other than HNO (reaction 7).

$$2 \text{ RC(O)NHO} \rightarrow \text{unidentified products}$$
(7)

The low yields of nitrite formed only during the electrolysis process could be attributed to RC(O)N=O oxidation to NO (reaction 8), which in aerated solutions yields nitrite.

$$RC(O)N=O + H_2O \rightarrow RC(O)O^- + NO + 2H^+ + e^-$$
(8)

Irrespective of the detailed mechanism, the relative long half-life of RC(O)N=O in aqueous solutions allows the study of its reactions with various nucleophiles as demonstrated below.

Reaction with amines

The decay of RC(O)N=O upon its reaction with various amines obeys first-order kinetics and the observed first order rate constant (k_{obs}) increases linearly upon increasing the concentrations of dimethylamine ($pK_a(-NH_3^+) = 10.73$), ethylamine ($pK_a(-NH_3^+) = 10.7$) or morpholine ($pK_a(-NH_3^+) = 8.36$) at constant pH (Figure 5A), and increases also as the pH increases (Figure 5B) implying that the reacting species is the basic form of the amine.

$$RC(O)N=O + R'NH_2 \rightarrow RC(O)NHR' + HNO$$
(9)



Figure 5. (A) Dependence of k_{obs} of the decay of RC(O)N=O derived from benzo-HX (\blacksquare), subero-HX (\blacktriangle) and SAHA (\bullet) on [dimethylamine] at pH 9.4; (B) Dependence of k_{obs} of the decay of CH₃C(O)N=O on [ethylamine]_o at pH 9.5 and 10.1. All solutions contained 0.1 M KCl and 50 mM borate buffer.

The apparent bimolecular rate constant (k_{app}) calculated from the slope of the lines such as those presented in Figure 5 are summarized in Table 4.

amine	pН	$C_5H_6C(O)N=O$	CH ₃ C(O)N=O	RC(O)N=O	RC(O)N=O
	_			from SAHA	from subero-HX
dimethylamine	7.2	0.28 ± 0.01	0.25 ± 0.01		
	7.8		0.39 ± 0.02		0.32 ± 0.02
	8.4		1.32 ± 0.08		
	9.2	13.2 ± 0.8	5.9 ± 0.3	4.7 ± 0.3	
	9.4				6.1 ± 0.2
	10.2		31.7 ± 1.8		
ethylamine	9.5		8.4 ± 0.6		
-	10.1		25.4 ± 0.9		
triethylamine	10.1		< 0.1		
morpholine	7.5		1.9 ± 0.1		
	9.4	12.0 ± 0.3	10.0 ± 0.6	4.4 ± 0.2	
A 11 1 /*	1011	VIZ 01 1 11 00	MANODO (1170		$\mathbf{D}\mathbf{C}$ ($\mathbf{I}\mathbf{I}\mathbf{O}$ 4) = C O

Table 4. The apparent bimolecular rate constant $(M^{-1}s^{-1})$ of the reaction of RC(O)N=O with amines.

All solutions contained 0.1 M KCl and either 20 mM MOPS (pH 7.2 - 7.8), 20 mM TAPS (pH 8.4) or 50 mM borate (pH 9.2 - 10.2) buffers.

In the case of CH₃C(O)N=O reaction with dimethylamine, a fit of k_{app} (Table 4) to a pK-curve using $pK_a(-NH_3^+) = 10.73$ resulted in $k_9 = 154 \pm 5 \text{ M}^{-1}\text{s}^{-1}$ assuming that the reaction with $(CH_3)_2NH_3^+$ is negligible. This value is similar to that obtained with ethylamine and is more than an order of magnitude higher than that with morpholine.

The rate of CH₃C(O)N=O decay at pH 10.1 (50 mM borate buffer) was unaffected by 10 - 60 mM triethylamine (p K_a (-NH₃⁺ = 11.0) implying that the reaction is too slow most probably due to steric hindrance.

Reaction with thiols

We have studied the reaction of RC(O)N=O with various thiols (R'SH) including cysteine $(pK_a(-SH) = 8.18, pK_a(-NH_3^+) = 10.36)^{30}$, GSH $(pK_a(-SH) = 8.7, pK_a(-NH_3^+) = 10.1)^{30}$ and NAC $(pK_a(-SH) = 9.51)^{30}$. The decay of RC(O)N=O obeys first order kinetics in the presence of R'SH and k_{obs} increases linearly upon increasing [R'SH] and increases also as the pH increases (Figure 6) implying that the reaction proceeds with R'S⁻ (reaction 10).

$$RC(O)N=O+R'S^{-} \rightarrow RC(O)SR'+NO^{-}$$
(10)



Figure 6. (A) Dependence of k_{obs} of the decay of CH₃C(O)N=O on [GSH] at pH 6.6 and 7.3; (B) Dependence of k_{obs} of the decay of C₅H₆C(O)N=O or CH₃C(O)N=O on [cysteine] at pH 6.6. All solutions contained 0.1 M KCl and 20 mM MOPS buffer.

The experiments were carried out at pH $\leq pK_a(-SH)$ since at higher pHs the rates become too fast for measurements under pseudo-first-order conditions using the current experimental setup. The k_{app} -values derived from the slope of the lines such as those in Figure 6 are summarized in Table 5.

thiol	рН	$C_5H_6C(O)N=O$	CH ₃ C(O)N=O	RC(O)N=O formed from	RC(O)N=O formed from	RC(O)N=O formed from
				SAHA	gly-HX	AABA-HX
cysteine	6.6	165 ± 9	56 ± 3			
	7.3		250 ± 15			
GSH	6.4				1150 ± 70	
	6.5					660 ± 70
	6.6	59 ± 2	27 ± 1	21 ± 1		
	7.3	240 ± 10	91 ± 3	77 ± 4		
NAC	6.4				172 ± 10	
	6.5					145 ± 10
	7.3	22 ± 1	9.5 ± 0.2	7.8 ± 0.1	535 ± 20	
	7.9	54 ± 3	37 ± 2	23 ± 1		

Table 5. The apparent bimolecular rate constant $(M^{-1}s^{-1})$ of the reaction of RC(O)N=O with thiols in 0.1 M KCl and 20 mM MOPS buffer.

Assuming that the rate of R'SH reaction with RC(O)N=O is negligible, one calculates $k_{10} = (6 - 8) \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ for C₅H₆C(O)N=O reaction with the thiolate ions of GSH, cysteine and NAC. In the case of CH₃C(O)N=O and the acyl nitroso derived from SAHA, k_{10} is about 2 - 3 folds lower. The acyl nitroso derived from gly-HX and AABA-HX at pH < 9 are positively charged, and this could be the reason for the relatively higher rate constant, *i.e.*, $k_{10} = (1 - 5) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$.

Reaction with hydroxamic acids

The rate of the first order decay of RC(O)N=O (k_{obs}) increases linearly upon increasing [HX] added following the oxidation and increases also as the pH increases (Figure 7). These results imply that RC(O)N=O reacts with X⁻ (reaction 11) and at constant pH the rate depends on the p K_a (-NHOH) of the reacting HX.

$$RC(O)N=O + RC(O)NHO^{-} \rightarrow RC(O)ONHC(O)R + NO^{-}$$
(11)



Figure 7. (A) Dependence of k_{obs} of the decay of CH₃C(O)N=O on [gly-HX] at pH 5.9, 6.6, 7.0 and 7.8; (B) Dependence of k_{obs} of the decay of C₅H₆C(O)N=O on [benzo-HX] at pH 7.3, 7.8 and 8.5. Solutions contained 0.1 M KCl and 20 mM of either MES (pH 5.9), MOPS (pH 6.6 – 7.8) or TAPS (pH 8.5) buffers.

The k_{app} -values calculated from the slope of the lines such as those presented in Figures 7 are summarized in Table 6.

	pН	aceto-HX	benzo-HX	gly-HX	lys-HX	DFO
	-	(9.0)	(8.54)	(7.4 - 7.8)	(7.9 - 8.0)	(8.7, 9.0, 10.1)
$CH_3C(O)N=O$	5.9			65 ± 5		
	6.2			69 ± 4		
	6.6			101 ± 8		
	7.0			169 ± 12		
	7.3	19 ± 2	49 ± 4	270 ± 18	356 ± 20	39 ± 2
	7.8	90 ± 7		414 ± 21		
	7.9			510 ± 18		
	8.5	303 ± 17		733 ± 36		352 ± 21
$C_6H_5C(O)N=O$	7.3	21 ± 1	36 ± 2			
	7.8		73 ± 5			
	8.5		268 ± 14			
RC(O)N=O	7.3	9.5 ± 1.0				
derived from						
subero-HX						

Table 6. The apparent bimolecular rate constant $(M^{-1}s^{-1})$ of RC(O)N=O reaction with HX at various pHs. In parenthesis are the $pK_a(-NHOH)$ of the reacting HX.

All solutions contained 0.1 M KCl and 20 mM of either MES (pH 5.9 - 6.2), MOPS (pH 6.6 - 7.9) or TAPS (pH 8.5) buffers.

Both gly-HX and DFO contain an amine group (Figure 1) where $pK_a(-NH_3^+) = 9.2^{22}$ and 8.4^{25} , respectively. In the case of gly-HX the highest studied pH 8.5 is lower than $pK_a(-NH_3^+) = 9.2$ thus eliminating the contribution of the amine to the reaction rate. A fit of k_{app} to a pK-curve resulted in $pK_a(-NHOH)) = 7.8 \pm 0.1$ and $k_{11} = 50 \pm 5$ and $860 \pm 40 \text{ M}^{-1}\text{s}^{-1}$ for the reaction of CH₃C(O)N=O with ⁺H₃NCH₂C(O)NHOH and ⁺H₃NCH₂C(O)NHO⁻, respectively. In the case of DFO the rate increases about an order of magnitude as the pH increases from 7.3 to 8.5, thus excluding the contribution of the amine moiety of DFO.

The decomposition of RC(O)N=O in the presence of excess aceto-HX yields N₂O. In fact, under the experimental conditions of Table 2 the yields of N₂O were unaffected by the presence of 2-3 mM aceto-HX. Since the reaction of RC(O)N=O with 2-3 mM aceto-HX efficiently competes with its hydrolysis, it is concluded that the reaction of RC(O)N=O with aceto-HX forms HNO (reaction 11).

HXs avidly chelate transition metal ions through the hydroxamate moiety,^{22,31} and therefore the question arises as to the effect of these chelates on the half-life of RC(O)N=O. The effect of metal ions including Cu^{II} , Ni^{II} , Zn^{II} and Co^{II} (denoted Me^{II}) on the decay of CH₃C(O)N=O has been

studied in the absence and presence of added aceto-HX at pH 7.3 (20 mM MOPS buffer). The most effective transition metal was Cu^{II}, which catalyzes efficiently the hydrolysis of CH₃C(O)N=O even in the absence of aceto-HX. Figure 8A shows that the catalysis rate constant ($k_{cat} = (1.5 \pm 0.7) \times 10^4$ M⁻¹s⁻¹ is practically the same whether electro-oxidation proceeds with 25 or 100 µM aceto-HX implying that the effect of residual unoxidized aceto-HX is negligible. This value decreased by about 50% in the presence of 2 - 8 µM Cu^{II} and 100 µM aceto-HX. Since at [aceto-HX]/[Cu^{II}] >10 the predominant complex at pH 7.3 is CuX₂,³² it is concluded that Cu^{II} alone is a better catalyst than CuX₂. In the case of all other transition metals studied, only the HX-metal complex has a significant effect on the decay rate of CH₃C(O)N=O (Figure 8B).



Figure 8. (A) Dependence of k_{obs} of the decay of CH₃C(O)N=O formed by electro-oxidation of 25 μ M aceto-HX (empty symbol) or 100 μ M aceto-HX (filled symbol) on [Cu^{II}] added following the electro-oxidation; (B) Dependence of k_{obs} of the decay of CH₃C(O)N=O formed by electro-oxidation of 100 μ M aceto-HX on [Me^{II}] and 150 μ M aceto-HX added following the electro-oxidation. All solutions contained 0.1 M KCl and 20 mM MOPS buffer at pH 7.3.

At high ratio of $[Me^{II}]/[HX]$, where the predominant species is MeX^+ , the catalysis rate was about twice higher than that of MeX_2 and/or MeX_3^- formed at high $[HX]/[Me^{II}]$ ratios (results not shown), implying that MeX^+ is a better catalyst. No rate increase of $CH_3C(O)N=O$ decomposition by 0.5 mM or 1 mM DFO-Fe^{III} was observed, whereas DFO alone does act as a nucleophile (Table 6). Thus, the metal binding to HX might affect the reaction rate either by catalyzing the hydrolysis of RC(O)N=O or by blocking HX nucleophilic activity.

Conclusions

Acyl nitroso species are formed in aqueous solutions *via* electro-oxidation of HXs, and undergo hydrolysis yielding mainly HNO and the respective carboxylic acid. Acyl nitroso species are characterized by their typical absorption at 314 - 330 nm and their relatively long half-lives allow for the first time the study of the kinetics of their reactions with various nucleophiles where the nucleophilic reactivity follows the order thiolate > hydroxamate > amine. Metal complexes of aceto-HX catalyze the hydrolysis of CH₃C(O)N=O at the efficacy order of Cu^{II} > Zn^{II} > Ni^{II} > Co^{II} where only Cu^{II} catalyzes the hydrolysis also in the absence of the hydroxamate Finally, oxidation of HXs can be used as a method for HNO generation where the half-life of this process is determined by that of the respective acyl nitroso species.

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Figure 2. Spectra of transient species formed upon electro-oxidation of HXs in aerated solutions containing 0.1 M KCl and 20 mM MOPS buffer at pH 7.3. Inset: first order kinetics of the decay monitored at λ_{max} . (A) 500 μ M acet-HX, $k_d = 0.19 \pm 0.01 \text{ min}^{-1}$; (B) 150 μ M benzo-HX, $k_d = 0.46 \pm 0.02 \text{ min}^{-1}$; (C) 230 μ M SAHA, $k_d = 0.14 \pm 0.01 \text{ min}^{-1}$; (D) 95 μ M subero-HX, $k_d = 0.21 \pm 0.01 \text{ min}^{-1}$.

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Figure 4. Spectra of transient species formed by electro-oxidation of 15 μ M AABA-HX (A) or gly-HX (B) in 0.1 M KCl and 20 mM MOPS buffer at pH 7.3. Inset: first-order decay kinetics of the transient species monitored at 325 nm, $k_d = 1.56 \pm 0.08 \text{ min}^{-1}$ (A) and at 317 nm, $k_d = 0.96 \pm 0.05 \text{ min}^{-1}$ (B).

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Figure 5. (A) Dependence of k_{obs} of the decay of RC(O)N=O derived from benzo-HX (\bullet), subero-HX (\diamond) and SAHA (\bullet) on [dimethylamine] at pH 9.4; (B) Dependence of k_{obs} of the decay of CH₃C(O)N=O on [ethylamine]o at pH 9.5 and 10.1. All solutions contained 0.1 M KCl and 50 mM borate buffer.

pH 9.5

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Figure 7. (A) Dependence of k_{obs} of the decay of CH₃C(O)N=O on [gly-HX] at pH 5.9, 6.6, 7.0 and 7.8; (B) Dependence of k_{obs} of the decay of C₅H₆C(O)N=O on [benzo-HX] at pH 7.3, 7.8 and 8.5. Solutions contained 0.1 M KCl and 20 mM of either MES (pH 5.9), MOPS (pH 6.6 – 7.8) or TAPS (pH 8.5) buffers.

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Figure 8. (A) Dependence of k_{obs} of the decay of CH₃C(O)N=O formed by electro-oxidation of 25 µM aceto-HX (empty symbol) or 100 µM aceto-HX (filled symbol) on [Cu^{II}] added following the electro-oxidation; (B) Dependence of k_{obs} of the decay of CH₃C(O)N=O formed by electro-oxidation of 100 µM aceto-HX on [Me^{II}] and 150 µM aceto-HX added following the electro-oxidation. All solutions contained 0.1 M KCl and 20 mM MOPS buffer at pH 7.3.

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