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Reactive oxygen species in aerobic decomposition of thiourea dioxides †

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Thiourea dioxides decompose in air-saturated alkaline solutions to give dithionite, $S_2O_4^{2-}$. Kinetics of decomposition of aminoiminomethanesulfinic acid (AIMSA), methylaminoiminomethanesulfinic acid (MAIMSA) and dimethylaminoiminomethanesulfinic acid (DMAIMSA) were studied in alkaline solutions under aerobic and anaerobic conditions. No dithionite was formed in strictly anaerobic conditions. Dithionite, however, was formed in the presence of KO_2 and H_2O_2 under anaerobic conditions. The rate of decomposition was fastest for DMAIMSA and slowest for MAIMSA. The proposed mechanism involves the initial formation of the dioxosulfate(2−) ion, SO_2^{2-}, through the heterolytic cleavage of the C–S bond. The dioxosulfate(2−) ion then reacts with dioxygen to give a series of reactive oxygen species: superoxide, peroxide and the hydroxyl radical. The expected dismutation of superoxide is important only in weakly alkaline solutions of pH less than 10. It is suggested, for the first time, that the reactive oxygen species and the sulfur leaving groups may be responsible for the toxicity observed in most thioureas.

Introduction

It is well known that many thioureas are toxins.[2,3] Substituted thioureas and thiourea itself are known to exhibit a number of pharmacological effects in toxicity and metabolic effects.[3] The main *in vivo* transformations of thioureas involve oxygenation of the sulfur atom followed by nucleophilic substitution or elimination reactions to give sulfinic and sulfonic acids.[4] It is known that S-oxygenation of thioureas results in the formation of genotoxic products.[5] The major products of oxidation, however, are the corresponding aminoiminomethanesulfinic acids (thiourea dioxides).[6] It was hypothesized that nucleophilic reactions of thiourea dioxides may be related to the reactions that lead to the toxicity of thioureas.[3] Animal studies on the chronic toxicity of thiourea have shown that when it is administered in drinking water it induces thyroid adenomas and carcinomas in rats.[7] The tumorigenicity of thiourea has been attributed to its strong antithyroid activity which leads to a disruption of the pituitary–thyroid hormonal regulatory system.[8] Thiourea inhibits thyroid peroxidase which results in an increased release of thyrotropin from the pituitary.[9]

Subtle differences in substituted thioureas can impart vastly different degrees of toxicity. For example, while α-naphthylthiourea produces pulmonary edema and pleural effusion in rats, its oxidation gives α-naphthylurea, an innocuous substance to rats.[10] In general, thioureas which substantially desulfurize during the metabolic processes, have been found to be the most toxic as is the case with α-naphthylthiourea. Thioureas that do not desulfurize and maintain a thione group as a metabolic end-product are generally non-toxic.[11] It is reasonable to assume, then, that the observed toxicity may arise from either the sulfur-containing leaving groups or the reactive oxygen species they generate.[12]

Surprisingly, there are no data available on toxic effects of inorganic sulfur-containing leaving groups released from thiourea dioxides in nucleophilic substitution/elimination reactions. Since these groups are strong reductants,[10] their reactions *in vivo* may potentially lead to the observed toxicity of thioureas. Here we report on the kinetic studies of aerobic decomposition of thiourea dioxide, *N*-methylthiourea dioxide, and *N*,*N*′-dimethylthiourea dioxide in aqueous solutions.

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Experimental

Materials

Aminoiminomethanesulfinic acid (AIMSA), methylurea, *N*,*N*′-dimethylurea, potassium superoxide, hydrogen peroxide, and α-isonitrosopropiophenone (ISPF) were purchased from Aldrich and used without further purification. Methylthiourea and 1,3-dimethyl-2-thiourea were purchased from Fluka and used as received. Superoxide dismutase (SOD) and catalase were purchased from Sigma.

Methylaminoiminomethanesulfinic acid (MAIMSA) was prepared according to a literature procedure by oxidation of methylthiourea with hydrogen peroxide.[13] *N*,*N*′-Dimethylaminoiminomethanesulfinic acid (DMAIMSA) was prepared by oxidation of 1,3-dimethyl-2-thiourea with hydrogen peroxide following the procedure used for the synthesis of MAIMSA.

Instrumentation

Conventional kinetics experiments were performed on a Perkin-Elmer Lambda 2S UV/Vis spectrophotometer. A Hi-Tech Scientific SF-DX2 stopped-flow spectrophotometer was used to measure induction times and to follow faster reactions. The products of MAIMSA and DMAIMSA decomposition were analyzed on a JNM GX-270 (JEOL) NMR spectrometer. GC-MS were measured with a Hewlett-Packard Model 5980 GC-MS using a mass selective detector.

Methods

All experiments were performed at 25 ± 0.2 °C. Temperature control was maintained with a NesLab RTE-101 thermostat. Reactions were run at an ionic strength of 0.5 M (NaCl). A mixture of acetic acid, phosphoric acid, and boric acid plus NaOH (Britton–Robinson system)[14] was used for the preparation of buffer solutions. In most reactions, however, a fixed

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0.5 M NaOH aqueous solution was used as the reaction medium. Decomposition of AIMSA and MAIMSA solutions was followed by measuring absorbance at 270 nm where the absorption coefficients of AIMSA and MAIMSA in water were found to be 489 and 521 M^{-1} cm^{-1} respectively. DMAIMSA decomposition was monitored by measuring absorbance at 263 nm where the absorption coefficient was found to be 475 M^{-1} cm^{-1}. Due to deprotonation of the sulfinic acids (pK_a-(AIMSA) = 8.01),[15] their absorption coefficients were higher at high pH conditions and hence had to be determined separately for solutions with different pH. Possible intermediates of aerobic decomposition of the sulfinic acids (superoxide, hydrogen peroxide and dithionite) also absorb significantly at 270 nm (ε = 1330, 110[16] and 2365 M^{-1} cm^{-1} respectively). Therefore we were unable to use experimental traces at 270 nm obtained in air-saturated solutions in our calculations of the rate constants at this wavelength. Dithionite formation was followed at 315 nm (ε = 8043 M^{-1} cm^{-1}).[17]

Urea, methylurea, and *N*,*N*′-dimethylurea were assayed with α-isonitrosopropiophenone (ISPF) reagent.[18] In control experiments AIMSA, MAIMSA, and DMAIMSA were assayed with ISPF, and none gave a positive test. The decomposition products of these sulfinic acids were also assayed with the ISPF reagent and all gave the distinctive pink color for the presence of corresponding ureas.

AIMSA did not show any reasonable NMR spectrum, and neither did its decomposition products. MAIMSA and DMAIMSA, however, each showed a single peak in each case representing the methyl protons. After decomposition, both MAIMSA and DMAIMSA yielded methylurea and *N*,*N*′-dimethylurea respectively. This was confirmed by comparing the chemical shifts of the methyl protons of authentic samples of methylurea and *N*,*N*′-dimethylurea with the decomposition products of MAIMSA and DMAIMSA under identical pH conditions. In addition, the presence of methylurea and *N*,*N*′-dimethylurea as the only detectable carbon-containing products of MAIMSA and DMAIMSA was independently confirmed by GC-MS. This shows that the decomposition of these sulfinic acids in alkaline conditions results in the cleavage of the C–S bond while leaving the rest of the organic backbone intact.

Results and discussion

Decomposition of air-saturated alkaline solutions of aminoiminomethanesulfinic acid (AIMSA), *N*-methylaminoiminomethanesulfinic acid (MAIMSA), and *N*,*N*′-dimethylaminoiminomethanesulfinic acid (DMAIMSA) is accompanied by the formation of dithionite $S_2O_4^{2-}$ (λ_{max} = 315 nm, ε_{315} = 8043 M^{-1} cm^{-1})[19] which is preceded by an induction period (Fig. 1A). Table 1 summarizes the major characteristics of the kinetic traces shown in Fig. 1A.

$S_2O_4^{2-}$ is known to exist in equilibrium with anion-radical $SO_2^{\bullet -}$.[19]

$$S_2O_4^{2-} \rightleftharpoons 2\ SO_2^{\bullet -} \quad (1)$$

The equilibrium of reaction (1) lies heavily to the right in aprotic solvents (CH_3CN, DMSO, *etc.*). In aqueous protic solutions the equlibrium constant has been quoted as being between 1.4×10^{-9} M and 1.4×10^{-6} M.[20]

Budanov and co-workers[21] explained the presence of dithionite in the alkaline decomposition of thiourea dioxide by the following reaction scheme that is initiated by the heterolytic cleavage of the C–S bond:

$$(NH_2)(HN{=})C{-}S(=O)OH + OH^- \rightleftharpoons (NH_2)_2CO + HSO_2^- \quad (2)$$

$$3\ HSO_2^- \rightleftharpoons 2\ HSO_3^- + HS^- \quad (3)$$

$$HSO_2^- + HSO_3^- \rightleftharpoons S_2O_4^{2-} + H_2O \quad (4)$$

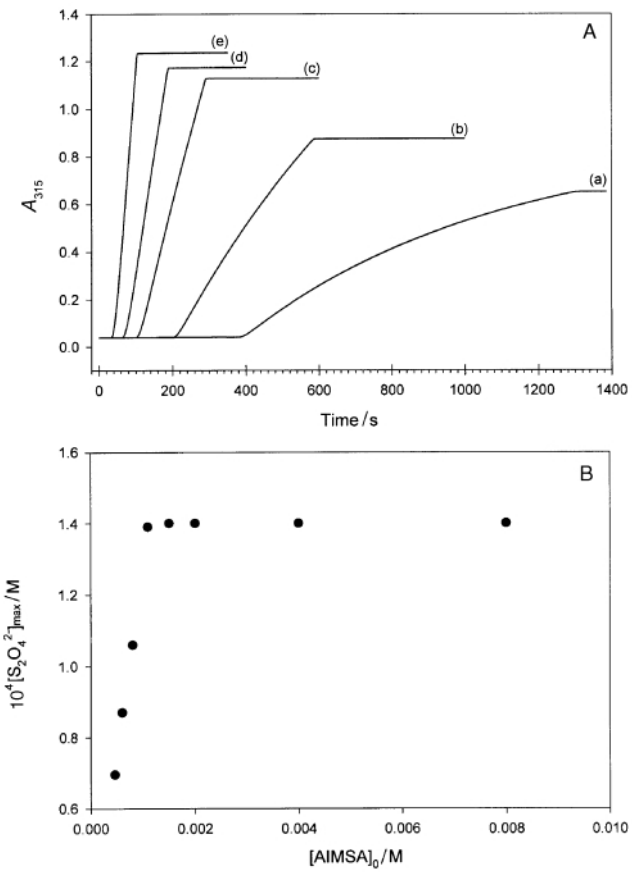

Fig. 1 (A) Formation of dithionite in the reaction of aerobic AIMSA decomposition in 0.5 M NaOH air-saturated solution. $[AIMSA]_0$ = (a) 0.6 mM; (b) 1 mM; (c) 3 mM; (d) 4.6 mM; (e) 6 mM. (B) Variation of the maximum dithionite concentration formed with respect to initial AIMSA concentrations. At high AIMSA concentrations the limiting factor is the amount of dissolved oxygen in solution, hence the saturation is observed.

This proposed mechanism, reactions (2)–(4), however, was not supported by our experimental data since we could not observe any influence of sulfite on the rate of dithionite formation in alkaline solutions. McGill and Lindstrom[22] assumed that the presence of the $SO_2^{\bullet -}$ anion-radical supports homolytic cleavage of the carbon–sulfur bond of AIMSA but they did not mention a possible role of oxygen in these processes.

By varying $[AIMSA]_0$ at fixed pH our experimental results showed that if $[AIMSA]_0 \gg [O_2]_0$ (with standard $[O_2]_0 = 2.4 \times 10^{-4}$ M in air-saturated aqueous solution and 2.15×10^{-4} M in 0.5 M NaOH),[23] the maximum concentration of $S_2O_4^{2-}$ attained during the reaction reached a constant value of about 1.4×10^{-4} M and did not depend on $[AIMSA]_0$ (Fig. 1B). In all our experiments, $[S_2O_4^{2-}]_{max}$ was found to be limited by the amount of oxygen initially present in solution. It was reasonable to assume, then, that oxygen was involved in the formation of $S_2O_4^{2-}$. Indeed, under rigorously anaerobic conditions dithionite was not formed at all. Further experimental data showed that oxygen did not affect the kinetics of AIMSA decomposition monitored at 270 nm. The 270 nm wavelength is the maximum in the absorption peak of AIMSA.

These results seem to suggest that dithionite and the anion-radical $SO_2^{\bullet -}$ are products of the reaction of oxygen with some sulfur-containing product of AIMSA decomposition. A possibility is the dioxosulfate(2−) ion, SO_2^{2-}, which is formed during heterolytic cleavage of the C–S bond.[24]

The reaction of SO_2^{2-} with O_2 is very fast and produces the $SO_2^{\bullet -}$ radical-ion:[25]

$$SO_2^{2-} + O_2 \longrightarrow SO_2^{\bullet -} + O_2^{\bullet -} \quad (5)$$

Table 1 Summary of the data presented in Fig. 1a

	[AIMSA]$_0$/M			
	1×10^{-3}	2×10^{-3}	3×10^{-3}	6×10^{-3}
Induction period/s	202	101	66	33
Zero-order rate[a]/M s^{-1}	2.67×10^{-7}	7.06×10^{-7}	1.12×10^{-6}	2.06×10^{-6}
$[S_2O_4^{2-}]_{max}$[a]/M	1.03×10^{-4}	1.35×10^{-4}	1.41×10^{-4}	1.48×10^{-4}

[a] Concentration of $S_2O_4^{2-}$ was calculated based on $\varepsilon_{315}(S_2O_4^{2-}) = 8043$ M^{-1} cm^{-1}.

The $SO_2^{\bullet -}$ radical-ion, in turn, is known to react with oxygen with a nearly diffusion-controlled rate constant of 2.4×10^9 M^{-1} s^{-1}:[26]

$$SO_2^{\bullet -} + O_2 \longrightarrow SO_2 + O_2^{\bullet -} \quad (6)$$

Both reactions (5) and (6) proceed during the induction period and produce the reactive oxygen species superoxide. If SO_2^{2-} and $SO_2^{\bullet -}$ were to react with oxygen alone one would not observe any formation of dithionite when all the oxygen in solution is consumed. Since formation of dithionite proceeds after the induction period there should be some reaction of SO_2^{2-} with $O_2^{\bullet -}$ or with the product of its dismutation: peroxide. Superoxide is known to be very stable in strongly alkaline solutions[27] and does not significantly dismutate in the time frame of our experiments. Therefore, we can assume that direct reaction of SO_2^{2-} and $SO_2^{\bullet -}$ with $O_2^{\bullet -}$ (accumulated during the induction period) commences after the induction period:

$$SO_2^{2-} + O_2^{\bullet -} \longrightarrow SO_2^{\bullet -} + O_2^{2-} \quad (7)$$

$$SO_2^{\bullet -} + O_2^{\bullet -} \longrightarrow SO_2 + O_2^{2-} \quad (8)$$

This conclusion has been confirmed by independent kinetic studies of the reaction between AIMSA and potassium superoxide (KO_2) under anaerobic conditions. Fig. 2A shows that kinetic traces at 315 nm resemble those obtained in air-saturated solutions without KO_2 (Fig. 1A). If $[KO_2]_0 = 0$ or $[KO_2]_0 \gg [AIMSA]_0$, dithionite is not formed at all (see trace (f)).

Reactions (7) and (8) produce another reactive oxygen species: peroxide. Peroxide's influence on the formation of $S_2O_4^{2-}$ in the absence of oxygen in strongly alkaline solutions is very similar to that of superoxide (Fig. 2B).[28] Stoichiometries of the reactions of SO_2^{2-} and $SO_2^{\bullet -}$ with peroxide are as follows:

$$SO_2^{2-} + O_2^{2-} + 2\,H_2O \longrightarrow SO_2^{\bullet -} + 3\,OH^- + OH^{\bullet} \quad (9)$$

$$SO_2^{\bullet -} + O_2^{2-} + 2\,H_2O \longrightarrow SO_2 + 3\,OH^- + OH^{\bullet} \quad (10)$$

Reactions (9) and (10) produce the most reactive oxygen species in the reaction sequence: the hydroxyl radical. It may disappear in a variety of pathways including the well-known, nearly diffusion-controlled reaction with sulfite $[SO_2(aq)]$[29] formed in reactions (6), (8), and (10). Indeed, addition of OH radical traps such as sodium formate or *tert*-butyl alcohol did not influence the kinetics of dithionite formation in the case of aerobic AIMSA decomposition.

In the absence of oxygen, we have found that the pseudo first-order rate constant for AIMSA decomposition in 0.5 M NaOH was 6.25×10^{-4} s^{-1}. A zero-order law for dithionite formation observed in Fig. 1A can be explained by the fact that in the time frame when actual dithionite formation takes place (linear rise) the concentration of AIMSA decreases rather insignificantly (*ca.* 10% in trace (b)). Therefore, it can be assumed that the rate of heterolysis of AIMSA to yield SO_2^{2-} (which then is very rapidly converted to SO_2^{-}) remains nearly constant. Indeed, when the time period of dithionite formation increases the deviation from zero-order kinetics becomes more and more evident (Fig. 1A).

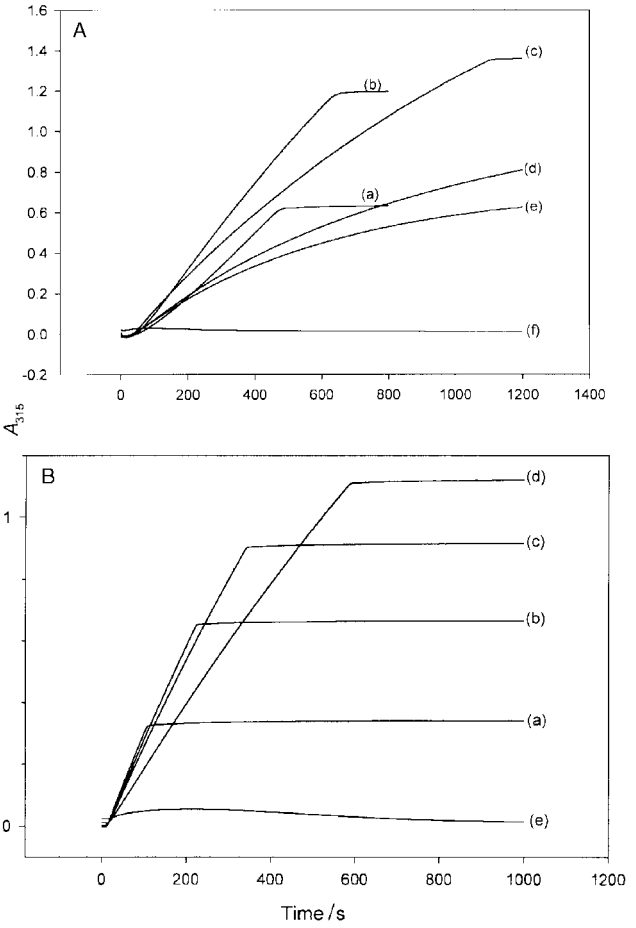

Fig. 2 (A) Formation of dithionite in the reaction of anaerobic decomposition of 1.2 mM AIMSA in 0.5 M NaOH in the presence of KO_2: (a) 0.1 mM; (b) 0.14 mM; (c) 0.35 mM; (d) 0.7 mM; (e) 7 mM. No dithionite was formed without KO_2. (B) Formation of dithionite in the reaction of anaerobic decomposition of 1.2 mM AIMSA in 0.5 M NaOH in the presence of H_2O_2: (a) 0.11 mM; (b) 0.22 mM; (c) 0.44 mM; (d) 0.88 mM; (e) 1.1 mM; (f) 4.4 mM. No dithionite was formed without H_2O_2.

At fixed $[O_2]_0$ and pH, the duration of the induction period depends solely on the initial concentration of AIMSA (Fig. 1A, Table 1). The data shown in Table 1 clearly indicate that higher concentrations of the substrate quickly form dithionite, and that there is a precise reciprocal relationship between concentration of substrate and the length of the induction period. This can be easily explained by invoking pseudo first-order (PFO) rate constant of AIMSA decomposition: $k_{obs} = [AIMSA]_0 \times k_{PFO}$, where $k_{PFO} = k[OH^-]$. Since k_{PFO} at $[OH^-] = 0.5$ M is constant (6.25×10^{-4} s^{-1}), k_{obs} will increase linearly with concentration of AIMSA. As a result, the time taken to consume all of the oxygen in solution will decrease reciprocally to k_{obs}. It is important to note that during the induction period every molecule of AIMSA is an origin for two oxygen consuming species, SO_2^{2-} and SO_2^{-}, hence the following relationship can be used to estimate the induction period (T): $T = [O_2]_0/2k_{PFO}[AIMSA]_0$. The calculated value (172 s for $[AIMSA]_0 =$

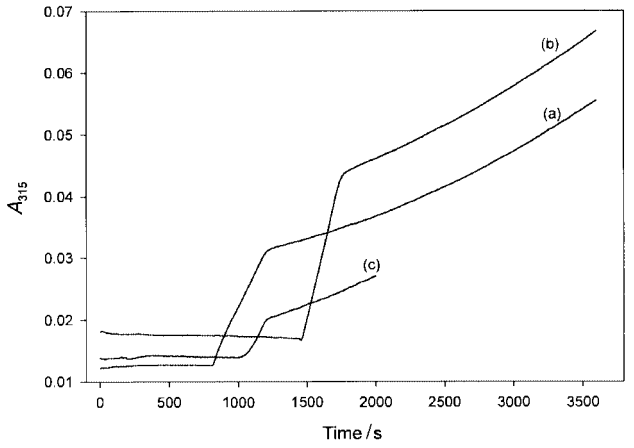

Fig. 3 Formation of dithionite during aerobic decomposition of 1.2 mM AIMSA in a buffer solution at pH 9.9: (a) without enzymes; (b) 1.5 μM SOD; (c) 0.125 μM catalase.

1 mM) is slightly lower than the experimental one, 202 s (Fig. 1A), due to partial contribution of superoxide to the consumption of SO_2^{2-} and SO_2^{-} during the induction period.

The mechanism of aerobic decomposition of AIMSA in weakly alkaline solutions is far more complex. Here, the dismutation of superoxide starts playing an important role.[29] This is reflected in the appearance of two distinct regions in the kinetic curves of dithionite formation (Fig. 3, trace (a)). Addition of superoxide dismutase (SOD) nearly doubled the induction period during the aerobic decomposition of AIMSA at pH 9.9 (Fig. 3, trace (b)). This effect can be explained by the increased concentration of oxygen in the solution due to rapid dismutation of $O_2^{\bullet -}$:[30]

$$2\ O_2^{\bullet -} + H_2O \longrightarrow O_2 + HO_2^{-} + OH^{-} \qquad (11)$$

Similar to SOD, addition of catalase increased the induction period due to formation of extraneous oxygen during peroxide dismutation (Fig. 3, trace (c)).

The maximum concentration of dithionite attained during the reaction in mildly alkaline solutions is much less than in strongly alkaline solutions. This is due to the increased reactivity of oxygen species formed in the aerobic decomposition process as well as the enhanced stability of AIMSA at pH near 7. But even in this case, reactions of SO_2^{2-} and $SO_2^{\bullet -}$ with O_2 will yield significant accumulation of toxic reactive oxygen species $O_2^{\bullet -}$, $OH^{\bullet}$ and O_2^{2-}.

All of the dependences mentioned above were also observed for MAIMSA and DMAIMSA. Measurements of the rates of decomposition of AIMSA, MAIMSA and DMAIMSA under anaerobic conditions revealed substantially different and structurally uncorrelated rate constants: k_{PFO} ($[OH^-] = 0.5$ M) = 6.25×10^{-4} s^{-1}; 1.56×10^{-4} s^{-1} and 5.04×10^{-3} s^{-1} respectively. Surprisingly, the rate of DMAIMSA heterolysis appeared to be significantly faster than that of both AIMSA and MAIMSA. The decomposition rate of MAIMSA was the slowest. Further investigations into the structural characteristics that might be accountable for the observed differences are in progress in our laboratories.

Conclusions

The results reported in this study show that the toxicity of thioureas might be partially attributed to *in vivo* reactions of SO_2^{2-} and $SO_2^{\bullet -}$ with oxygen resulting in the production of a series of reactive oxygen species: $O_2^{\bullet -}$, O^{2-}, and $OH^{\bullet}$.

These reactive oxygen species, especially the hydroxyl radical, can cause DNA damage. In some of our previous work,[31] our experiments on the decomposition of hydroxymethanesulfinic acid showed significant production of the sulfite anion-radical which can cause substantial damage to DNA.[32] Further work in this area will include an extension of these kinetic and mechanistic studies to other thiourea dioxides and nucleophiles.

Acknowledgements

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