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Superoxide, Amine Buffers and Tetranitromethane: A Novel Free Radical Chain Reaction

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The amine buffer Tris slowly reduces tetranitromethane (TNM) to the nitroform anion in a non-accelerating reaction. The amine buffers HEPES and MOPS also (slowly) react with TNM but the dialkylaminoalkyl radicals formed from these two buffers undergo further reactions resulting in a rapid, accelerating, free radical chain process whereby the amine is oxidized and TNM reduced. The chemical functionality in *any* reaction component, not necessarily the buffer, required for this radical chain mechanism is >N-CH<. In the presence of such groups, the quantification of superoxide by TNM is impossible.

Keywords: amine buffer; radical chain reaction; superoxide; tetranitromethane

INTRODUCTION

The superoxide radical anion, $O_2^{\bullet-}$, plays a central role in oxidative stress.[1] As *ex vivo* studies of oxidative stress become more probing and sophisticated, the importance of unequivocally identifying the agent inducing the stress and determining the precise flux of the agent generated has become ever more apparent. Superoxide fluxes are commonly quantified by UV-Vis spectrophotometry using Fe^{III} cytochrome c or tetranitromethane (TNM) which, on reduction by $O_2^{\bullet-}$, show grow-in absorptions with $\lambda_{max} = 550$ and 350 nm, respectively. During a recent study of the utility and reliability of these two $O_2^{\bullet-}$ probes[2] we discovered an interesting, $O_2^{\bullet-}$ mediated, free radical chain reaction between TNM and some of the more popular amine buffers. It is hoped that this brief report will prevent others from attempting to quantify $O_2^{\bullet-}$ formation in amine buffers using TNM.

MATERIALS AND METHODS

Chemicals

The following materials were purchased and used as received: TNM (Aldrich); Tris, MOPS and HEPES (Sigma); water (Millipore). The structures of Tris, MOPS and HEPES are shown in Fig. 1. Di-(4-carboxybenzyl)hyponitrite (SOTS-1) was synthesized as previously described.[3]

Methods

All reactions were carried out at 37 °C and pH 7.4 in air-saturated aqueous solutions made from

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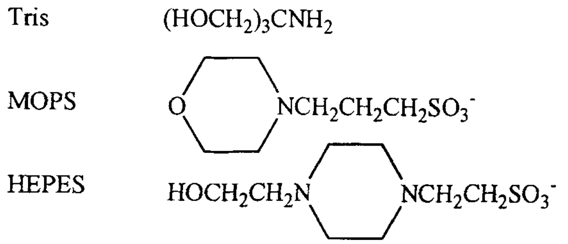

FIGURE 1

doubly-distilled water which was also treated with Chelex-100 for at least 24 h before use to remove or, at least to greatly reduce, the concentration of contaminating transition metal ions. Changes in absorbance were monitored using a Hewlett Packard 8452A diode array spectrophotometer and analyzed using HP 98531A UV-Vis operating software.

RESULTS

The reaction of superoxide with TNM is diffusion-controlled ($k_1 = 2 \times 10^9$ M^{-1} s^{-1}[4]) and yields the nitroform anion which absorbs strongly at 350 nm ($\varepsilon = 15,000$ M^{-1} cm^{-1}[5]).

$$\underset{\text{TNM}}{O_2{}^{\bullet -} + C(NO_2)_4} \longrightarrow O_2 + NO_2{}^{\bullet} + {}^{-}C(NO_2)_3 \tag{1}$$

The nitroform anion is also formed from TNM in aqueous solution via base-catalyzed hydrolysis.

$$HO^- + C(NO_2)_4 \longrightarrow H^+ + NO_3{}^- + {}^-C(NO_2)_3 \tag{2}$$

However, this reaction is extremely slow at 37°C and pH 7.4. At this pH, the rate of nitroform anion formation from TNM (100 µM) is faster in 50 mM Tris buffer than in 50 mM phosphate buffer, see inset in Figure 2. The Tris-induced nitroform anion formation is a bimolecular reaction ($k = 3 \times 10^{-3}$ M^{-1} s^{-1}). There is, therefore, a slow direct reaction between Tris and TNM which could be mistaken for superoxide formation, but this reaction is not autocatalytic. More interesting are the auto-accelerating reactions of TNM (100 µM) with 50 mM of either MOPS or HEPES (see Figure 2). The lag phase for MOPS (ca. 1 min) is much shorter than for HEPES (ca. 8 min) and the subsequent formation of nitroform anion is considerably faster for MOPS than HEPES. The long lag phase with HEPES means that a brief (≤ 5 or 6 min) "control" experiment with TNM and HEPES buffer would lead to the incorrect conclusion that this probe / buffer system could safely be used to measure superoxide formation from a source of superoxide.

DISCUSSION

The chemistry which is probably responsible for the autocatalytic formation of the nitroform anion from TNM in MOPS or HEPES buffer was adumbrated by Kirsch *et al.*'s[6] discovery that under free radical conditions HEPES reacts with dioxygen to give superoxide. This discovery was itself foreshadowed by the report Das et al.[7] that in aqueous solution the dimethylaminomethyl radical reacts with dioxygen to form superoxide.

$$(CH_3)_2NCH_2{}^{\bullet} + O_2 \longrightarrow (CH_3)_2\overset{+}{N}{=}CH_2 + O_2{}^{\bullet -} \tag{3}$$

The structures of MOPS and HEPES imply that their one electron oxidation would yield dialkylaminoalkyl radicals and these, of course, would react with dioxygen to form superoxide. For thermodynamic reasons, the superoxide radical anion could not abstract a hydrogen atom from a trialkylamine. Even for the hydroperoxyl radical ($HOO^{\bullet}$), which at pH 7.4 is present at 0.25% of the superoxide, this reaction will be very slow (k probably <10 M^{-1} s^{-1}; see [8]). In the presence of TNM all the superoxide will undergo reaction (1) to form the strong 1-electron oxidizing agent, nitrogen dioxide, which is capable of oxidizing alkylamines.[9] Thus, all the necessary conditions are met for an accelerating radical chain reaction when TNM is added to aerated MOPS or HEPES buffer. First, a slow, continuous source of radicals to initiate the chain:

$$C(NO_2)_4 + (-CH_2)_2\overset{\bullet\bullet}{N}CH_2- \longrightarrow NO_2^{\bullet} + {}^{-}C(NO_2)_3 + (-CH_2)_2\overset{\bullet+}{N}CH_2- \qquad (4)$$

Second, a series of fast reactions which propagate the chain but, remarkably, do not consume dioxygen:

$$NO_2^{\bullet} + (-CH_2)_2\overset{\bullet\bullet}{N}CH_2- \longrightarrow NO_2^{-} + (-CH_2)_2\overset{\bullet+}{N}CH_2- \qquad (5)$$

$$(-CH_2)_2\overset{\bullet+}{N}CH_2- \xrightarrow{-H^+} (-CH_2)_2\overset{\bullet\bullet}{N}\overset{\bullet}{C}H- \qquad (6)$$

$$O_2 + (-CH_2)_2\overset{\bullet\bullet}{N}\overset{\bullet}{C}H- \longrightarrow O_2^{\bullet-} + (-CH_2)_2\overset{+}{N}{=}CH- \qquad (7)$$

$$O_2^{\bullet-} + C(NO_2)_4 \longrightarrow O_2 + NO_2^{\bullet} + {}^{-}C(NO_2)_3 \qquad (1)$$

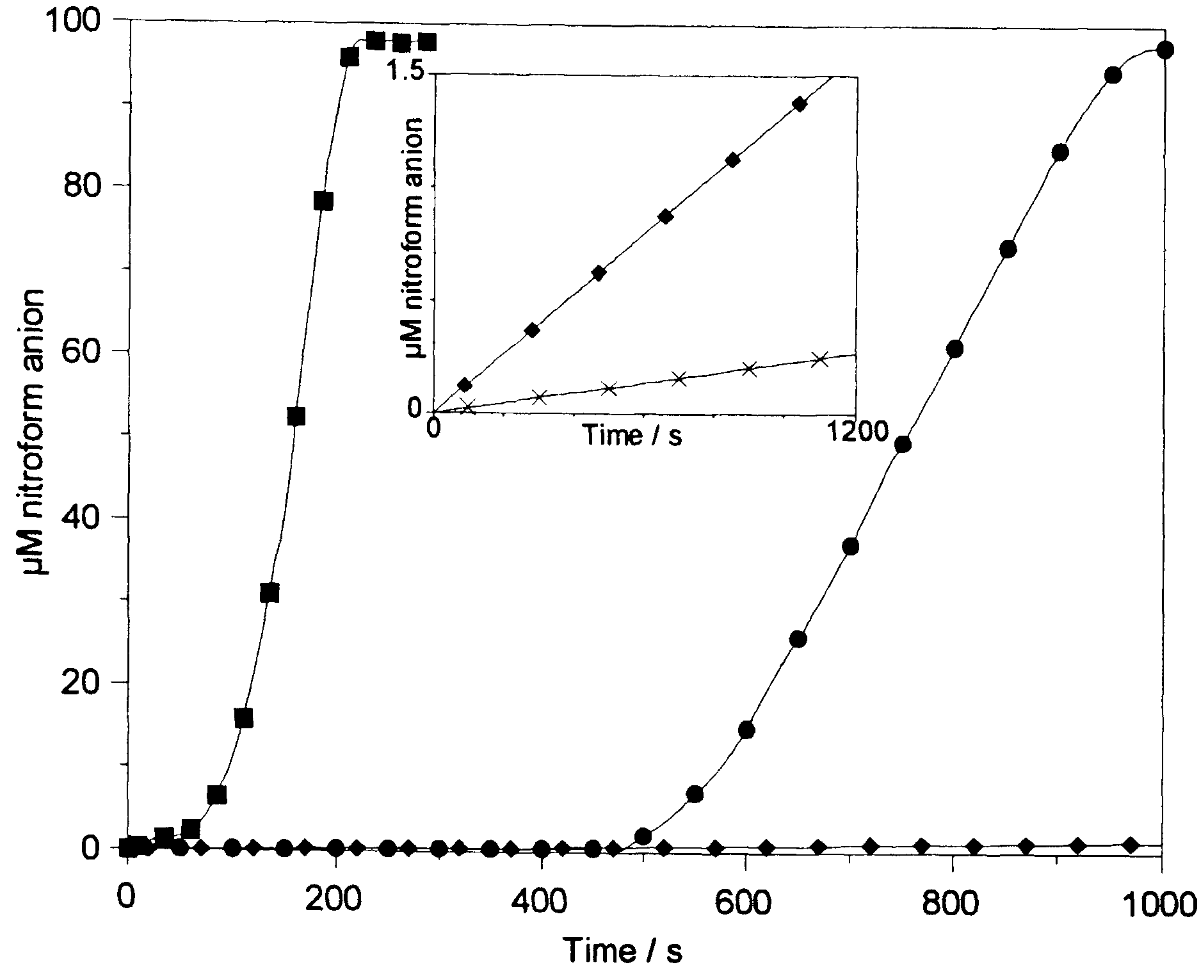

FIGURE 2 Formation of the nitroform anion by reaction of TNM (100 µM) with some common amine buffers (all at 50 mM) in aerated aqueous solution at 37°C and pH 7.4. Key: ■, MOPS; •, HEPES; ♦, Tris. Inset: Tris and phosphate (50 mM, X) on an expanded scale

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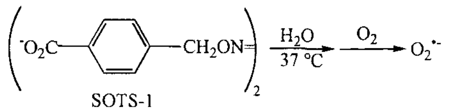
The lag phase is presumably due to the slow buildup of $NO_2^\bullet$ and $(-CH_2)_2\overset{\bullet+}{N}CH_2-$ to their steady-state concentrations. Because reaction (1) is diffusion-controlled, chain termination is unlikely to involve $O_2^{\bullet-}$. The most probable chain termination process is:

$$2NO_2^\bullet \rightleftharpoons N_2O_4 \xrightarrow{H_2O} NO_2^- + NO_3^- + 2H^+ \quad (8)$$

In support of the above chain reaction mechanism, the lag phase with MOPS and HEPES was completely eliminated by the addition of our clean, chemical, thermal source of superoxide, SOTS-1[3], even at levels where $O_2^{\bullet-}$ was generated at a rate of only 4 μM/min. Under these conditions, initiation by the slow reaction 4 is replaced by initiation via reactions (9) and (1).



SOTS-1

(9)

It will be obvious that the chain reaction shown above cannot occur with Tris because this buffer lacks the necessary >N-CH< moiety (see Fig. 1). However, it will occur in aerated TNM-containing systems which contain molecules (not necessarily added as buffers) which do contain the >N-CH< moiety. If the concentration of such molecules is low, the lag phase will be prolonged, but the chain reaction will eventually occur.

We trust that this cautionary note will prevent others from experiencing our own confusion when we first encountered this phenomenon.

Acknowledgements

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