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## An Analysis of the Effect of Carbon Dioxide on the Decay Rates of Superoxide

Michael Jay Walsh  
*Colby College*

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**An Analysis of the Effect of Carbon Dioxide  
on the Decay Rates of Superoxide**

Michael Jay Walsh

A Thesis Presented to the Department of Chemistry,  
Colby College, Waterville, ME  
In Partial Fulfillment of the Requirements for Graduation  
With Honors in Chemistry

Submitted May 18, 2005

**An Analysis of the Effect of Carbon Dioxide  
on the Decay Rates of Superoxide**

By Michael Jay Walsh

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## Table of Contents

List of Figures	5
List of Tables	6
Acknowledgements	7
Abstract	8
Introduction	9
Materials and Methods	18
Results and Discussions	40
Conclusions	47
Future Work	47
Works Cited	50

## List of Figures

<b>Figure 1:</b> Second order decay rate as a function of pH.	11
<b>Figure 2:</b> First order decay rates of superoxide	14
<b>Figure 3:</b> Fraction of carbonate by pH	19
<b>Figure 4:</b> Flow chart of experiment.	22
<b>Figure 5:</b> Production of superoxide by photolysis of benzophenone	24
<b>Figure 6:</b> Photolizer and UV/Vis cuvette holder	26
<b>Figure 7:</b> Photolysis and decay of a superoxide containing solution	27
<b>Figure 8:</b> Absorbance decay curve of superoxide.	31
<b>Figure 9:</b> Gas delivery system	33
<b>Figure 10:</b> Gas mass flow controller	34
<b>Figure 11:</b> Gas mass flow controller circuit	35
<b>Figure 12:</b> Methylated Cypridina Luciferin Analogue (MCLA)	36
<b>Figure 13:</b> Superoxide detection system	37
<b>Figure 14:</b> Decay of superoxide in various systems	41
<b>Figure 15:</b> First order decay rates of superoxide by carbonic acid concentration	43
<b>Figure 16:</b> First order decay rates of superoxide	45

## List of Tables

<b>Table 1:</b> Selected decay rates	12
<b>Table 2:</b> First order decay rates in various systems	42

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## Abstract

The radical dioxygen anion, superoxide has been shown to react with carbon dioxide in nonprotic and aqueous systems. Indirect evidence suggests that superoxide also reacts with carbon dioxide and carbonate species dissolved in water. This study involved conducting experiments to determine the decay rate of superoxide in the presence of carbon dioxide and carbonate species. Experiments were conducted by sparging an alkaline bicarbonate buffer with carbon dioxide and measuring the decay rate of superoxide added to the solution. Although the specific reaction mechanism and rates have not been determined first order superoxide decay rates as high as  $3.8 \times 10^{-2} \text{ s}^{-1}$  were obtained and an increasing trend in the rates was found with the increase of carbonic acid. Contamination from impurities and the inability to make a control solution made the exact determination and quantification of rates and mechanisms impossible at this time. This study laid the groundwork needed for the development of a robust mechanistic model for the decay of superoxide by reaction with carbon dioxide.

## Introduction

Photochemically generated oxygen species play an integral role in many environmental and biological systems as intermediates in redox chemistry. Of these species the radical anion superoxide,  $O_2^{\bullet-}$ , has been shown to be active in many processes including redox reactions with metals and organics as well as the primary precursor to hydrogen peroxide.

Superoxide is found in many biological systems as a byproduct of metabolic processes in mitochondria. The molecule becomes a deleterious free radical and is a dangerous oxidant that damages enzymes, membranes, and nucleic acids by peroxidating such molecules. The enzyme Superoxide Dismutase (SOD) is present in many organisms and is responsible for facilitating the removal of superoxide.

Superoxide is present in natural marine environments at steady state concentrations of  $10^{-7}$  to  $10^{-9}$  Molar<sup>1</sup> and may possibly play an influential role in the biogeochemistry of marine phytoplankton. Kustka et al. suggested that superoxide is produced by marine phytoplankton to reduce Iron (III) to Iron (II), the biologically available form of iron.<sup>2</sup> It has also been suggested that superoxide may be produced by marine phytoplankton to ward off harmful bacteria because of its toxic properties.

In aquatic systems the primary source of superoxide is from the photolysis of Chromophoric Dissolved Organic Matter (CDOM). During this process UV sunlight excites CDOM to a triplet state. Excited CDOM reacts with dissolved oxygen to form the superoxide radical.



The primary sink of superoxide, well documented by Bielski et. al., is the disproportionation reaction of superoxide with itself or its conjugate acid to form hydrogen peroxide<sup>3</sup>:



Because  $\text{HO}_2^-$  is a weak acid with a  $pK_a$  of 4.69, the overall superoxide decay will depend on a pH weighted sum of the rates of reactions (4) and (5) shown by equation (6).

$$k_d = \frac{\left( k_{\text{HO}_2 + \text{HO}_2} + k_{\text{HO}_2 + \text{O}_2^-} \left( \frac{K_a}{[\text{H}^+]} \right) \right)}{\left( 1 + \frac{K_a}{[\text{H}^+]} \right)^2} \quad (6)$$

Values for the rate constants  $k_{\text{HO}_2 + \text{HO}_2}$  and  $k_{\text{HO}_2 + \text{O}_2^-}$  can be found in Table 1. The pH dependence of the second order rate constant is shown by Figure 1. As long as the rate constant  $k_d$  is used for the disproportionation rate expression  $\text{O}_2^{\cdot-}$  is equivalent to the total superoxide concentration consisting of  $\text{HO}_2^{\cdot}$  and  $\text{O}_2^{\cdot-}$ .

Another major sink of superoxide in marine systems is dissolved transition metals. For example, both Fe(II) and Fe(III) will react with superoxide.<sup>1, 4</sup>



The presence of such metals catalyzes the reaction 1,000 fold for the rapid, of superoxide in comparison to the disproportionation. Rate constants for (7), (8) and other reactions can be found in Table 1.

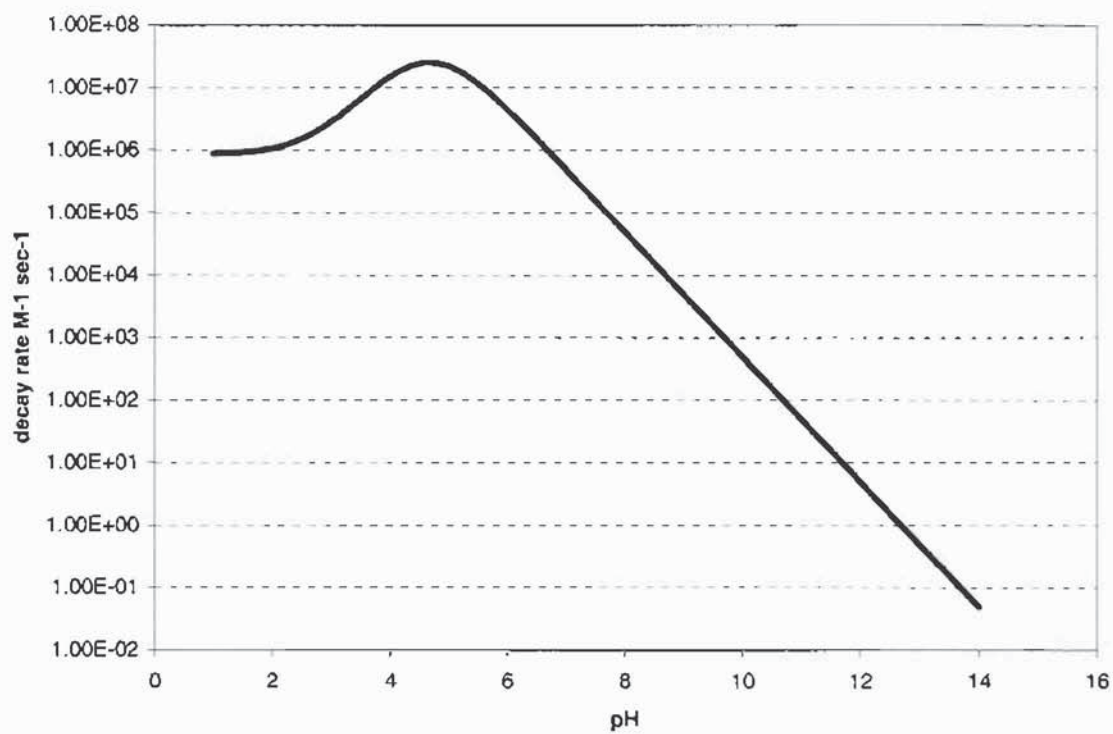


Figure 1. Second order decay rate of superoxide as a function of pH. Calculated from Equation (6)

Table 1. Specific second order decay rates of superoxide and various metals. Adapted from reference <sup>5</sup>.

Reaction	Reaction Equation	Rate Constant (M <sup>-1</sup> s <sup>-1</sup> )	Reference
3	$H^+ + O_2^- \rightarrow HO_2$	$1.60 \times 10^{-5}$	3
4	$HO_2 + HO_2 \rightarrow H_2O_2 + O_2$	$8.30 \times 10^{+5}$	3
5	$HO_2 + O_2^- \rightarrow HO_2^- + O_2$	$9.70 \times 10^{+7}$	3
7	$Fe(II) + O_2^- + H^+ \rightarrow Fe(III) + HO_2^-$	$1.00 \times 10^{+7}$	4
8	$Fe(III) + O_2^- \rightarrow Fe(II) + O_2$	$1.50 \times 10^{+8}$	4
	$Fe(II) + HO_2 + H^+ \rightarrow Fe(III) + H_2O_2$	$1.20 \times 10^{+6}$	4
	$Fe(III) + HO_2 \rightarrow Fe(II) + H^+ + O_2$	$<1.00 \times 10^{+3}$	4
	$Cu(I) + O_2^- + 2H^+ \rightarrow Cu(II) + H_2O_2$	$1.00 \times 10^{+9}$	6
	$Cu(I) + O_2^- \rightarrow Cu(II) + OH^- + HO_2^-$	$1.00 \times 10^{+10}$	6
	$Cu(II) + \rightarrow H^+ + Cu(I) + O_2$	$1.00 \times 10^{+8}$	6
	$Cu(II) + O_2^- \rightarrow Cu(I) + O_2$	$5-8.00 \times 10^{+9}$	6

Petasne & Zika suggested the presence of an unknown pathway for superoxide disappearance in seawater in addition to the disproportionation reaction and aside from the presence of metals.



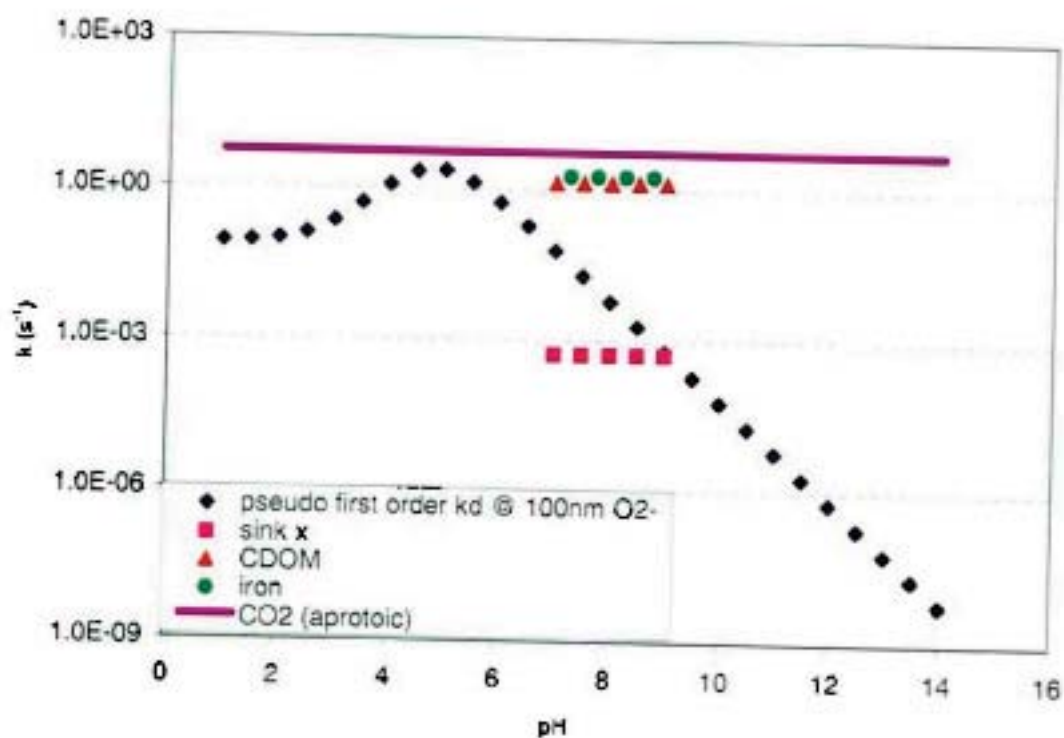
The unknown sink showed a first order decay giving a combined decay first order and second order decay.<sup>7</sup>

$$\frac{d[O_2^{\cdot-}]}{dt} = k_d[O_2^{\cdot-}]^2 + k_x[O_2^{\cdot-}] \quad (10)$$

The rates of  $k_x$  were demonstrated to be as high as  $5.4 \times 10^{-4} \text{ sec}^{-1}$ . Such a sink will not result in the production of HOOH. As a result of this, it is possible to quantify the disproportionation reaction as well as the unknown sink simultaneously by analyzing HOOH concentration at the same time as measuring superoxide.

In addition to being a source for superoxide, CDOM also serves as a sink. Goldstone and Voelker demonstrated that superoxide in costal seawater shows a first order decay with rates as high as  $1.4 \text{ s}^{-1}$ , which they attribute to reactions with CDOM.<sup>8</sup> One could assume that CDOM was responsible for the first order decay of superoxide noticed by Petasne & Zika. However, the decay constants are much different suggesting different mechanisms.

The reactivity of superoxide by first and second order decay processes may be compared at specific superoxide concentrations by converting second order rates to pseudo first order rates using an assumed concentration of superoxide. These results are shown in Figure 2 allowing for a comparison of all decay processes. For example, the relative effect of iron can also be examined using this method. The rate laws for iron mediated superoxide decay are:



*Figure 2.* Pseudo first order decay rates of superoxide. The diamonds represent the pseudo first order decay rate obtained by multiplying  $k_d$  by 100nM of superoxide. The squares are sink  $x$  at  $5.4 \times 10^{-4} s^{-1}$ . The triangles represent CDOM's decay rate of  $1.4 s^{-1}$ , and iron's pseudo first order decay at  $0.94 s^{-1}$  for 100nm total iron in natural systems. The straight line is the decay rate of superoxide as a result of carbon dioxide in aprotic solvents at  $5.5 s^{-1}$ .

$$\frac{d[\text{O}_2^{\bullet-}]}{dt} = \frac{d[\text{Fe(II)}]}{dt} = k_7[\text{Fe(II)}][\text{O}_2^{\bullet-}] \quad (11)$$

and

$$\frac{d[\text{O}_2^{\bullet-}]}{dt} = \frac{d[\text{Fe(III)}]}{dt} = k_8[\text{Fe(III)}][\text{O}_2^{\bullet-}] \quad (12)$$

At steady state these two rate laws are equal giving:

$$k_7[\text{Fe(II)}] = k_8[\text{Fe(III)}] \quad (13)$$

This relationship results in a ratio of 15:1 for Fe(II) to Fe(III) based on rates  $k_7$  and  $k_8$ .

Assuming a total iron concentration of 100nm then there is approximately 94nm of Fe(II). The pseudo first order superoxide loss rate for iron can now be calculated by summing up the two decays,

$$\frac{d[\text{O}_2^{\bullet-}]}{dt} = k_7[9.4 \times 10^{-8}][\text{O}_2^{\bullet-}] + k_8[6.3 \times 10^{-9}][\text{O}_2^{\bullet-}] = 1.88 \text{ sec}^{-1}[\text{O}_2^{\bullet-}] \quad (14)$$

In addition to the species described above, superoxide has been shown to react with carbon dioxide. Schmidt proposed a mechanism for the elimination of superoxide in an aqueous carbonate system mediated by bicarbonate<sup>9</sup>:



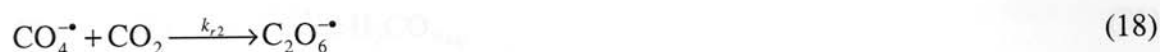
However this mechanism fails to take into account reactions with other carbonate species and dissolved  $\text{CO}_2$ . It is also unlikely because of charge repulsion of the two negative species making a reaction unfavorable. It does provide evidence that superoxide does decay in the presence of a carbonate species or  $\text{CO}_2$ .



The carbon dioxide reaction has been well documented in non-aqueous solutions. Roberts et. al. proposed a mechanism in which superoxide nucleophilically attacks carbon dioxide in the aprotic solvents; dimethyl sulfoxide, dimethylformamide and acetonitrile.<sup>10</sup> This reversible process produces a peroxy carbonate radical anion,  $\text{CO}_4^{\bullet-}$  in its initial step:



This reaction was shown to be first order with respect to superoxide in all solvents and showed rates for  $k_{r1}$  to be between 2.6 and  $5.5\text{s}^{-1}$  which is depicted in Figure 2. The peroxy carbonate radical is consumed by the following steps



A rate for carbon dioxide mediated superoxide decay in aqueous systems should be between this rate and the Petasne & Zika rate of  $5.4 \times 10^{-4}$  and this study will look for a rate in that range.

Indirect evidence for superoxide reactivity with carbon dioxide in aqueous solutions has been observed in the luminol-superoxide reaction. Xiao et. al. have shown that luminol chemiluminescence requires the presence of a peroxy carbonate anion radical, seen in Roberts' study. The authors proposed that the radical is produced by the reaction of carbon dioxide with superoxide produced from the analyte. In related experiments carbon dioxide quenched the reaction between 1,10-phenanthroline and superoxide. Presumably, carbon dioxide added to the system scavenges the superoxide preventing reaction with 1,10-phenanthroline.<sup>11</sup>

## Carbonate System

This study was specifically concerned with the reaction of superoxide and dissolved carbon dioxide gas,  $CO_{2(aq)}$ . Atmospheric  $CO_2$  has a partial pressure of  $3.55 \times 10^{-4}$  atm (preindustrial  $pCO_2 = 2.9 \times 10^{-4}$  atm) accounting for 0.0335% of the volume of air at 1 atm.<sup>12</sup> In aqueous systems carbon dioxide dissolves in water and equilibrates with the carbonate system, a diprotic system involving carbonic acid, bicarbonate and carbonate. The gas exchange equilibrium is defined by Henry's Law for carbon dioxide.



The hydration equilibrium for carbon dioxide to carbonic acid is



Of all the equilibria in the carbon dioxide/carbonate system, (21) is slowest on a time scale of minutes. By convention, the acid form of the carbonate system is described as the sum of dissolved  $CO_2$  and carbonic acid.



where  $H_2CO_3^*$  is representative of all  $H_2CO_{3(aq)}$  and  $CO_{2(aq)}$ . The hydration equilibrium of carbon dioxide gas can now be described as



where  $K_g$  is defined by

$$K_g = K_H(1 + K) \quad (24)$$

The acid base reactions of this system are:



The acid base distribution of the carbonate system is controlled by the following fractions,  $\alpha_i$ :

$$\alpha_{H_2CO_3^*} = \frac{[H^+]^2}{[H^+]^2 + K_{a1}[H^+] + K_{a1}K_{a2}} \quad (27)$$

$$\alpha_{HCO_3^-} = \frac{K_{a1}[H^+]}{[H^+]^2 + K_{a1}[H^+] + K_{a1}K_{a2}} \quad (28)$$

$$\alpha_{CO_3^{2-}} = \frac{K_{a1}K_{a2}}{[H^+]^2 + K_{a1}[H^+] + K_{a1}K_{a2}} \quad (29)$$

Where  $K_{a1}$  is  $4.47 \times 10^{-7}$  and  $K_{a2}$  is  $4.68 \times 10^{-11}$  for pure water at  $25^\circ\text{C}$ . The distribution as a function of pH is shown by Figure 3.

Another way to describe the carbonate system is using the concept of alkalinity.

Where the presence of  $H^+$  ions is considered acidity, alkalinity is the measure of the deficit of  $H^+$  ions in a carbonate system defined by

$$[Alk] = [HCO_3^-] + 2[CO_3^{2-}] \quad (30)$$

By fixing the alkalinity of a solution by employing known carbonate or bicarbonate concentration and controlling the  $pCO_2$ , the amount of dissolved carbon dioxide as well as carbonic acid in solution can be determined and controlled. The concentration of  $H_2CO_{3(aq)}^*$  can be determined from the total alkalinity and the fraction of  $H_2CO_{3(aq)}^*$  using equations (25), (26), (27) where  $\beta$  is the charge and mole balanced fraction of the respective species.

$$[H_2CO_{3(aq)}^*] = [Alk]\beta_{H_2CO_3} = \frac{[Alk][H^+]^2}{K_{a1}[H^+] + K_{a1}K_{a2}} \quad (31)$$

$$[HCO_{3(aq)}^-] = [Alk]\beta_{HCO_3^-} = \frac{[Alk]K_{a1}[H^+]}{K_{a1}[H^+] + K_{a1}K_{a2}} \quad (32)$$

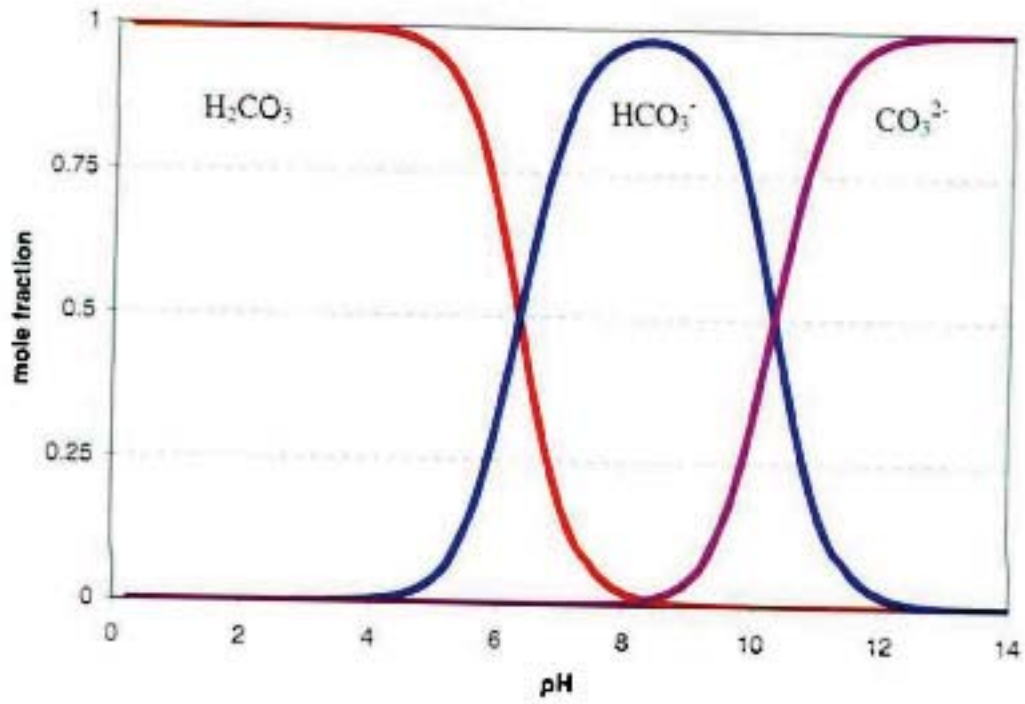


Figure 3. Fraction of carbonate species by pH. Natural systems vary from pH 7 to pH 8.5 resulting in a dominance of bicarbonate.

$$[CO_{3(aq)}^-] = [Alk]\beta_{CO_3^-} = \frac{[Alk]K_{a1}K_{a2}}{K_{a1}[H^+] + K_{a1}K_{a2}} \quad (33)$$

The goal of this study is to quantify the effect of carbon dioxide on the decay rates of superoxide in aqueous systems. This will be done measuring superoxide decay rates in solutions with well defined CO<sub>2</sub> and carbonate concentrations. Ideally, from this study a mechanistic model for superoxide decay in the presence of CO<sub>2</sub> and carbonate may be developed.

## Materials and Methods

### *Reagents*

Milli-Q water (18M $\Omega$ ) was used to prepare all solutions. All reagents were obtained from Sigma-Aldrich and used as received, except for MCLA which was obtained from Molecular Probes. Gases were obtained from BOC Gases.

### *pH Measurements*

The pH was carefully measured using an Orion Thermo Sure Flow pH electrode model 9165BN and a Fisher Scientific Accumet pH/Conductivity meter calibrated daily using a 3-point NIST standardization, using commercially available buffers.

### *Experiments*

This experimental design utilized two apparatuses. The first was a spectrophotometric system used to determine the initial concentration of superoxide at micromolar concentrations. The second apparatus was a chemiluminescence (CL) instrument used to determine the concentrations of superoxide at nanomolar concentrations with and without added CO<sub>2</sub> or carbonate.

The flow of experiments is shown by Figure 4. Buffered sample solutions were prepared before each experiment through which the desired gas was bubbled using a gas flow control system. Superoxide stock was made using literature methods and analyzed spectrophotometrically to determine the concentration. A specific volume of superoxide was then added to the sample solutions. The sample solution was pumped into a superoxide CL detection system which quantified superoxide concentration. A decay curve was fit to this data and first and second order rate constants obtained using least squares curve fitting.

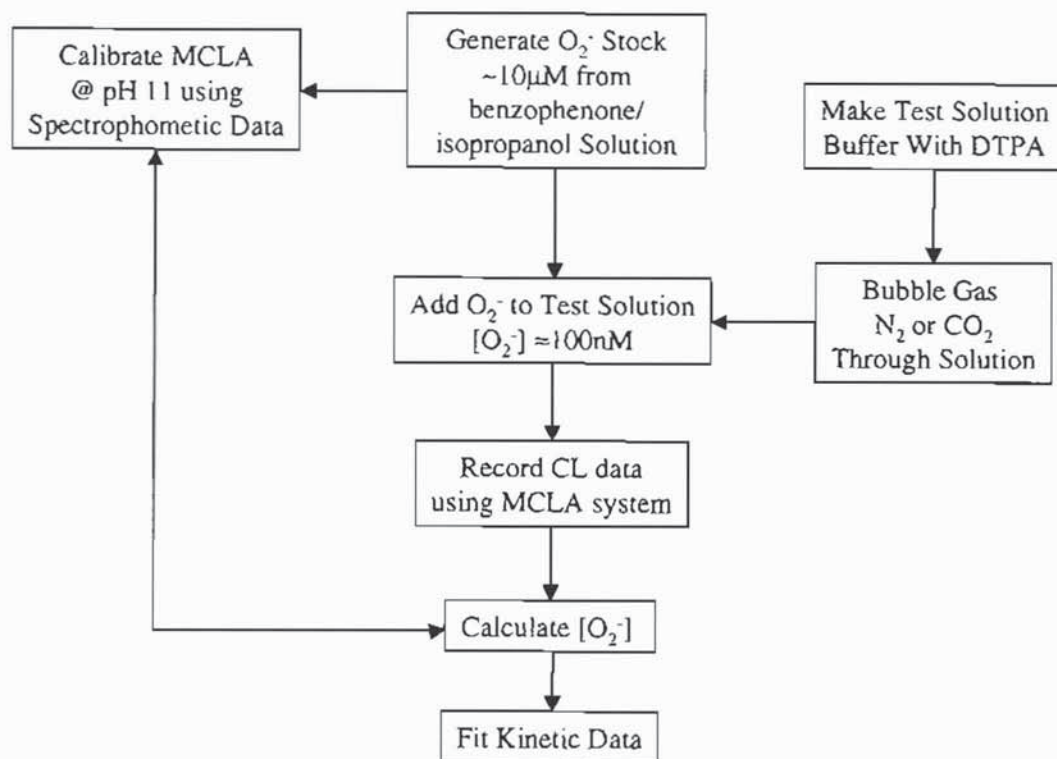


Figure 4. Flow chart of experiment.

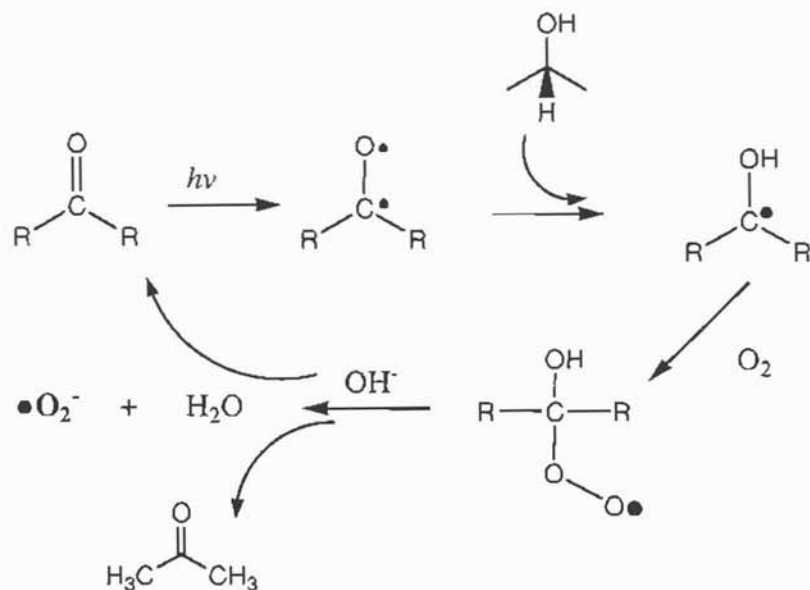
### *Production of a Superoxide Stock*

Quantifiable, suitably concentrated stock solutions of superoxide are needed for analysis. Commercial superoxide sources, such as  $\text{KO}_2$  are unviable for our purposes, because of their impurity and high metal contamination. In the past, superoxide has been most often produced by a complex pulse radiolysis method. A quick and easy method has been suggested by McDowell to mimic the production of superoxide by CDOM. This method involves exciting a  $n-\pi^*$  triplet state of a ketone and quenching the excited ketone with a secondary alcohol to form a radical, which then reduces dissolved oxygen to form superoxide as shown by Figure 5. Such a reaction can be carried out using a UV lamp. The benzophenone is regenerated in this reaction and isopropanol oxidized to a ketone. Both of these two organic compounds have been shown not to have a significant effect on the decay of superoxide.<sup>13</sup>

Superoxide is an extremely reactive species and can readily react with many dissolved trace metals in solution as shown in Figure 2. For this study it is necessary to remove metal species that would react with superoxide. A metal chelator reduces the activity of metals. The 6- coordinate ligand EDTA would be the expected choice, but superoxide appears to react with the metal ligand complex. The seven coordinate DTPA is an effective chelator that has been shown not to have any effect on the decay rate of superoxide in natural waters.<sup>14</sup>

The photolysis method of McDowell<sup>13</sup> was utilized to produce superoxide. The solution, contained 200 $\mu\text{M}$  DTPA, 40mM 2-propanol and 0.005M benzophenone and was buffered to a pH of 11 using a 0.73mM borate buffer with 2mM sodium hydroxide.



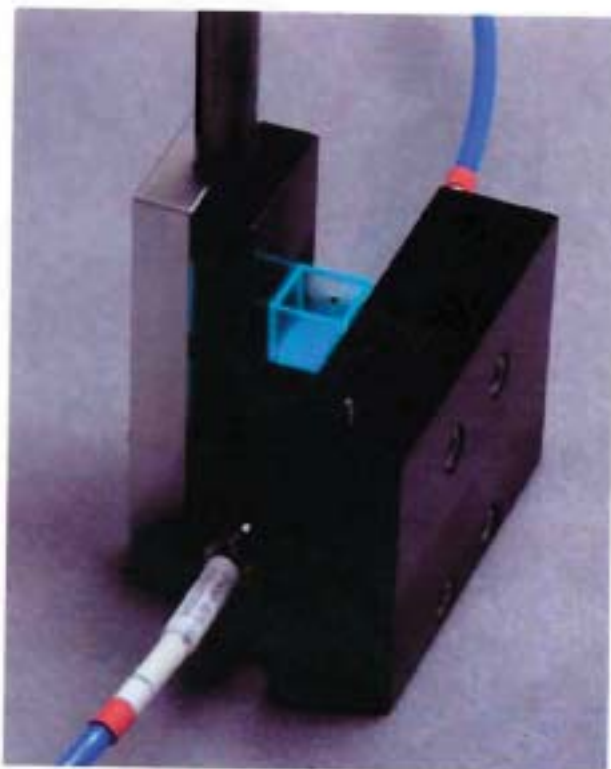


*Figure 5.* Production of Superoxide by Photolysis of Benzophenone. A ketone was photolyzed under basic conditions to form a  $n-\pi^*$  triplet state. The excited molecule was quenched by a primary or secondary alcohol; in this experiment isopropanol was used. The solution was saturated with oxygen to induce superoxide formation when oxygen reacts with the radical ketone. The ketone is reformed and isopropanol is oxidized to acetone.

### *Photolysis and Spectroscopic Analysis of Superoxide*

A cuvette holder (Figure 6) that served as a simultaneous irradiation source and UV/Vis cell was engineered to employ a UVP Pen Ray mercury UV pen lamp as a photolysis source, and provide a maximum amount of light to the cuvette. The output of the lamp at 240nm is  $1.20 \times 10^{-7}$  Einsteins  $s^{-1} m^{-2}$ . The cuvette holder is also equipped with a water cooled heat sink since continuous running of the pen lamp raises the solution temperature. The length of photolysis in these experiments was too short to generate a significant amount of heat.

The concentration of superoxide was measured spectrophotometrically, using an Ocean Optics USB2000 Spectrophotometer with an Ocean Optics MiniD2T Deuterium Tungsten source. Approximately 3ml of unphotolyzed solution was placed in a 10mm square Fisherbrand quartz fluorescence cuvette which was placed in the holder and the blank measured. Four clear wall fluorescence cuvettes were used because a clear third wall orthogonal to the spectrophotometric path was needed for UV light irradiation. The holder sat on a magnetic stir plate and the solution was stirred with a small stir bar. Photolysis began after 30 seconds of data collection on the solution absorbance blank and continued as desired. Data was collected until decay effectively stopped. This setup worked very well for the simultaneous production and spectrophotometric quantification of superoxide. Data from a typical experiment is shown in Figure 7.



*Figure 6.* Photolizer and UV/Vis Cuvette Cell. The blue fiber optics are the optical path of the light for the UV/VIS spectrophotometer. Orthogonal to the UV/VIS path is the UV Hg pen lamp illuminating the sample in a 3ml cuvette. The cell is geometrically designed so that the pen lamp has no effect on the absorbance determination.

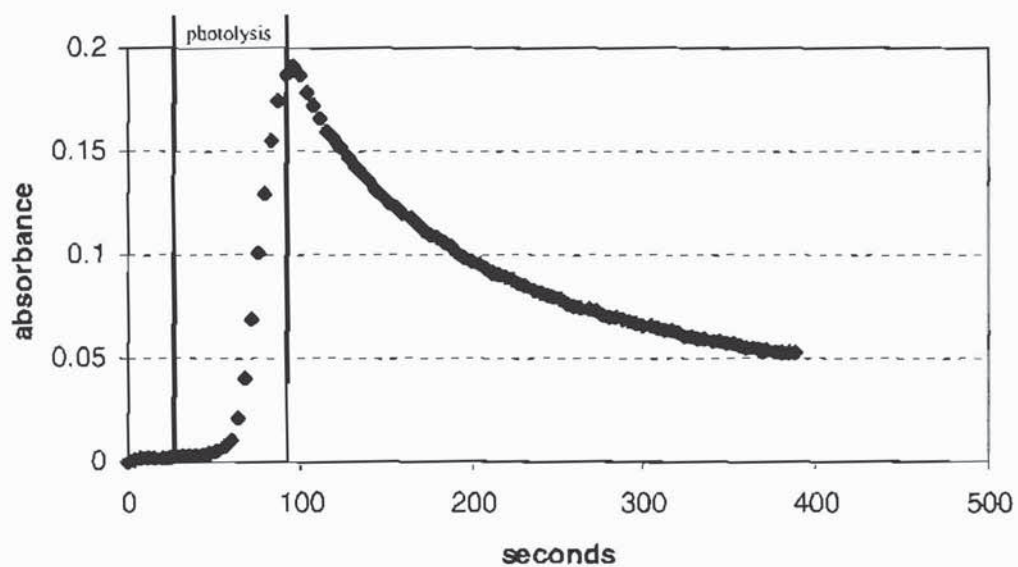


Figure 7. Absorbance of a superoxide containing solution over time. Photolysis, represented by the region between the two vertical lines, begins at 30 seconds and runs for 60 seconds.

The concentration of superoxide during the photolysis can be calculated from absorbance measurements using its molar extinction coefficient. However the two species,  $O_2^-$  and  $HO_2$  have similar, but different absorbance spectra. Bielski reported the  $\lambda_{max}$  for these two species to be 225nm ( $\epsilon_{max} = 1400 \pm 80 M^{-1} cm^{-1}$ ) for  $HO_2$  and 245nm ( $\epsilon_{max} = 2350 \pm 120 M^{-1} cm^{-1}$ ) for  $O_2^-$ .<sup>15</sup> Also due to the disproportionation reaction the superoxide produces hydrogen peroxide, which also has distinct spectra both for its protonated and unprotonated species. At any time, t, in the experiment, the total absorbance is a sum of the superoxide and peroxide absorbances.

$$A_t = ([O_2^*]_t \epsilon_{HO_2+O_2}^* + [HOOH]_t \epsilon_{HOOH+HOO^-}^*) \ell \quad (34)$$

The effective molar extinction constant of superoxide is a weighted sum of the extinction constants of the protonated and unprotonated forms of superoxide.

$$\epsilon_{HO_2+O_2}^* = \frac{[HO_2]}{[O_2^*] + [HO_2]} \epsilon_{HO_2} + \frac{[O_2^*]}{[O_2^*] + [HO_2]} \epsilon_{O_2^-}, \quad (35)$$

which can be calculated as a function of pH:

$$\epsilon_{HO_2+O_2}^* = \frac{[H^+]}{K_{a(HO_2)} + [H^+]} \epsilon_{HO_2} + \frac{K_{a(HO_2)}}{K_{a(HO_2)} + [H^+]} \epsilon_{O_2^-} \quad (36)$$

The pH dependant speciation of hydrogen peroxide follows a similar relationship:

$$\epsilon_{HOOH+HOO^-}^* = \frac{[HOOH]}{[HOOH] + [HOO^-]} \epsilon_{HOOH} + \frac{[HOO^-]}{[HOOH] + [HOO^-]} \epsilon_{HOO^-} \quad (37)$$

which can also be calculated as a function of pH

$$\epsilon_{HOOH+HOO^-}^* = \frac{[H^+]}{K_{a(HOOH)} + [H^+]} \epsilon_{HOOH} + \frac{K_{a(HOOH)}}{K_{a(HOOH)} + [H^+]} \epsilon_{HOO^-} \quad (38)$$

The combined  $\lambda_{max}$  of superoxide in aqueous solution for a pH greater 8 is 240nm where  $\epsilon_{240} = 2345$ . Since all spectrophotometric data was obtained at a pH of 11 this

wavelength will be used for our calculations. At this wavelength the molar absorptivities for hydrogen peroxide are  $\epsilon_{240} = 31 \text{ M}^{-1} \text{ cm}^{-1}$  for the protonated form and  $\epsilon_{240} = 320 \text{ M}^{-1} \text{ cm}^{-1}$  for the deprotonated form.

Photolysis shown in Figure 7 was conducted for 60 seconds. During this time the production of superoxide is much greater than that of hydrogen peroxide because the production rate is much larger than the decay rate. Considering the lower molar absorptivities of HOOH, the absorbance of HOOH is negligible at the start of the experiment resulting in the absorbance proportional to only the superoxide concentration.

$$[O_2^{\bullet-}]_o = \frac{A_o}{\epsilon_{HO_2+O_2^-}} \quad (39)$$

where  $[O_2^{\bullet-}]$  represents the total superoxide concentration, protonated and deprotonated forms.

Disproportionation of superoxide to HOOH must be considered after photolysis because the reaction times are long enough to allow significant decay of superoxide to produce HOOH. At any time during the decay, mass balance requires that the initial superoxide concentration equals to the superoxide concentration plus twice the peroxide concentration. Using the stoichiometric ratios from equations (3) and (4):

$$[O_2^{\bullet-}]_o = [O_2^{\bullet-}]_t + 2[HO_2H]_t \quad (40)$$

Thus the hydrogen peroxide concentration can be expressed as

$$[HO_2H]_t = \frac{[O_2^{\bullet-}]_o - [O_2^{\bullet-}]_t}{2} \quad (41)$$

Including equations (40) and (41) into equation (34) results in the absorbance as a function of time,  $A_t$  gives:

$$A_t = \left( [O_2^{\bullet-}]_t \varepsilon_{HO_2+O_2^-}^* + \frac{1}{2} \left( \frac{A_o}{\varepsilon_{HO_2+O_2^-}^*} - [O_2^{\bullet-}]_t \right) \varepsilon_{HOOH+HOO^-}^* \right) \ell \quad (42)$$

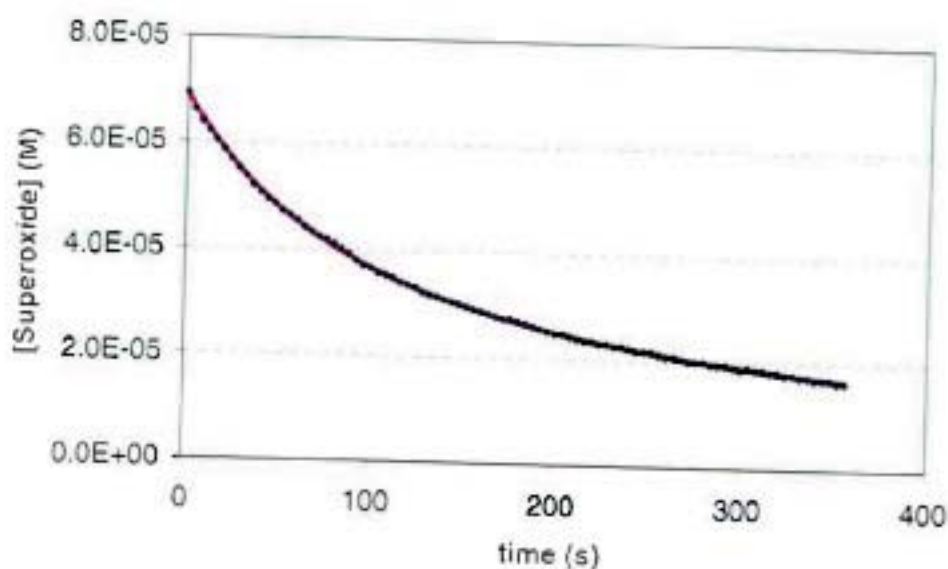
Which can be rearranged:

$$A_t - \frac{1}{2} A_o \left( \frac{\varepsilon_{HOOH+HOO^-}^*}{\varepsilon_{HO_2+O_2^-}^*} \right) \ell = [O_2^{\bullet-}]_t \left( \varepsilon_{HO_2+O_2^-}^* - \frac{1}{2} \varepsilon_{HOOH+HOO^-}^* \right) \ell \quad (43)$$

At low pH the term  $-\frac{1}{2} A_o \left( \frac{\varepsilon_{HOOH+HOO^-}^*}{\varepsilon_{HO_2+O_2^-}^*} \right) \ell$  is  $<1\%$ ; then equation (43) becomes:

$$\frac{A_t}{[O_2^{\bullet-}]_t} = \varepsilon_{\text{effective}} = \left( \varepsilon_{HO_2+O_2^-}^* - \frac{1}{2} \varepsilon_{HOOH+HOO^-}^* \right) \ell \quad (44)$$

At higher pH's ( $>5$ ) the correction term gets larger,  $\sim 5\%$ , and equation (43) should be used. Since the pH of the superoxide stock solution ranged between 11 and 12, equation (43) was used to calculate the total concentration of superoxide. A second order exponential curve was fit to the decay data (Figure 8) using the least squares method. From this fit, concentrations of superoxide and hydrogen peroxide were calculated as a function of time. The concentration data versus time also allowed calculation of the second order rate of superoxide in the stock solution. This allowed us to confirm that the decay of the stock solution was following known decay rates. Superoxide was produced between  $50\mu\text{M}$  and  $100\mu\text{M}$ .



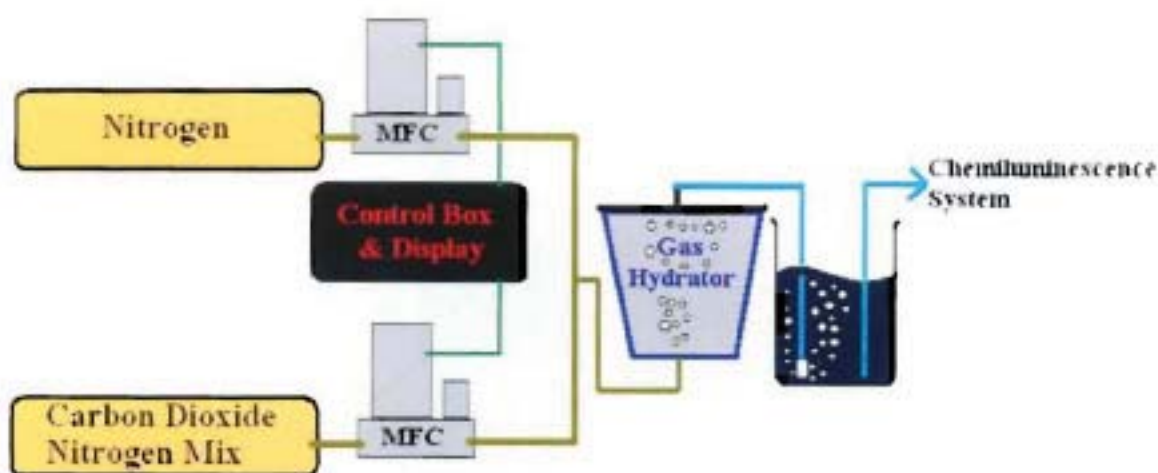
*Figure 8.* Decay curve of superoxide over time at pH 11.3. The data is fitted with a second order decay curve. The rate constant obtained from the fit is comparable with known rates at this pH. The initial concentration of superoxide in the decay provides the initial stock of superoxide for subsequent experiments.



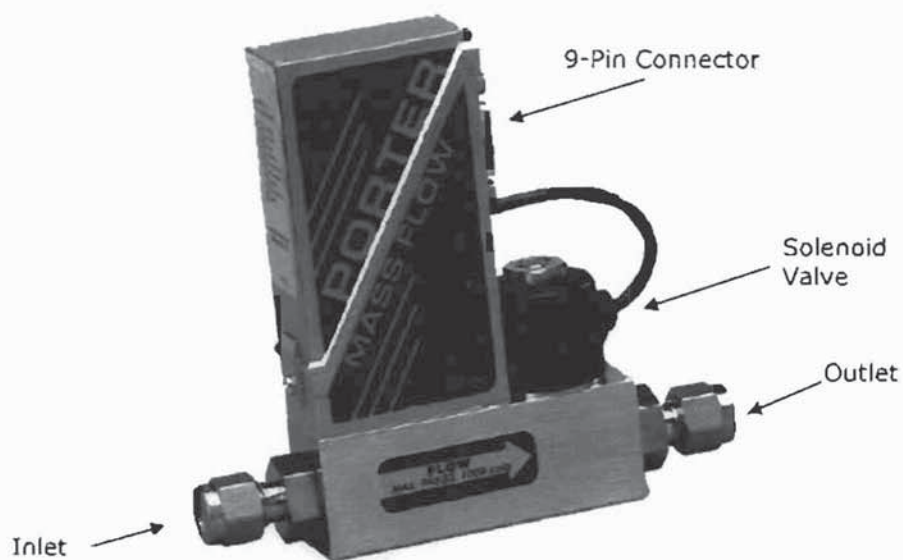
### *Gas Mass Flow Controllers*

A gas delivery system was utilized to control the  $p\text{CO}_2$  and thus the amount of dissolved carbon dioxide in the system (Figure 9). The system utilizes gas mass flow controllers (Figure 10), MFC's, to deliver a desired amount of different gasses. MFC's utilize the thermal absorption or specific heat capacity of gasses to measure flow. As a gas is heated the temperature increases at a rate proportional to its mass. Knowing the temperature increase over a specific time interval, it is possible to measure the flow of mass over time. Our system uses a bypass tube with an upstream thermistor, a downstream thermistor and a heater between the two. The thermistors and heater are connected in a Wheatstone Bridge Circuit. The flow controller consists of a solenoid valve that opens and shuts to control the flow. The solenoid is adjusted so that the actual gas flow measured with the sensor matches the desired gas flow set by the set point controller on the control circuit (Figure 11).<sup>16</sup>

Each mass flow controller comes with a specified maximum flow rate measured in standard cubic centimeters per minute (SCCM) or standard liters per minute (SLCM). The MFC's were controlled by applying a reference voltage (0-5 volts) proportional to the total flow of the devices. Two mass flow controllers can be used in unison to adjust the partial pressure of a specific gas. This task is preformed by linking the outputs of two mass flow controllers for different gasses or gas mixtures with a T-connector.



*Figure 9.* Gas delivery system. A nitrogen tank and a 2% carbon dioxide (98%  $N_2$ ) tank were each connected to a gas mass flow controller (Figure 10) using plastic tubing. The two mass flow controllers were governed by a circuit (Figure 11) placed in a control box. The output of the MFC's were linked to a T-connector using copper tubing. The gases are mixed in the T-connector and passed through a gas hydrator. The purpose of the hydrator is to prevent evaporation of the solution by saturating the gas stream with water. A gas frit delivers the gas to solution.



*Figure 10.* Gas Mass Flow Controller (MFC). The solenoid valve controlled the flow of gas through the MFC. A 9-pin connector was linked to the control box (circuit shown in Figure 6), which powered and operated the MFC.

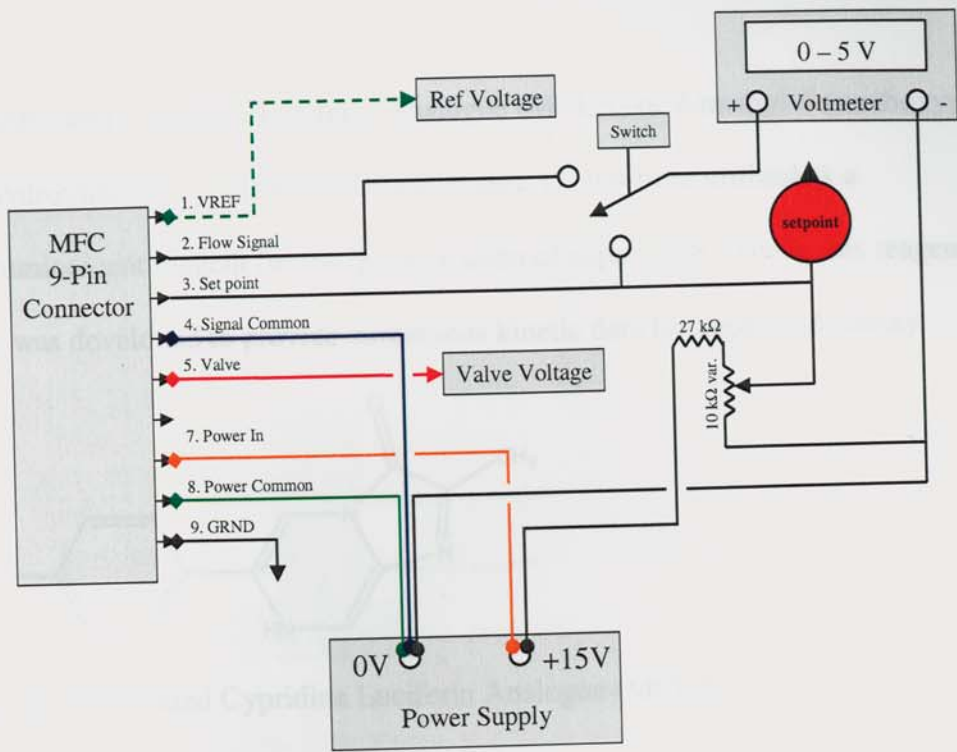


Figure 11. Gas Mass Flow Controller Circuit. This Circuit was built for each gas mass flow controller used. The circuit was powered by a 15V DC power supply. The flow could be controlled by turning the Setpoint dial controlling a variable 10kΩ resistor. Actual flow could be measured by flipping a switch to the controller flow signal output. The voltmeter measures the potential on a scale of 0 to 5 volts. The flow can be found by multiplying the proportion of the maximum voltage (5V) by the designated maximum flow of the MFC. (Adapted from reference <sup>16</sup>)

## MCLA

The Methylated Cypridina Luciferin Analogue (MCLA) or 2-methyl-6-(methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one, Figure 12, has been utilized as a chemiluminescent reagent for the quantification of superoxide.<sup>5</sup> Using this reagent a system was developed to provide continuous kinetic data for superoxide decay.

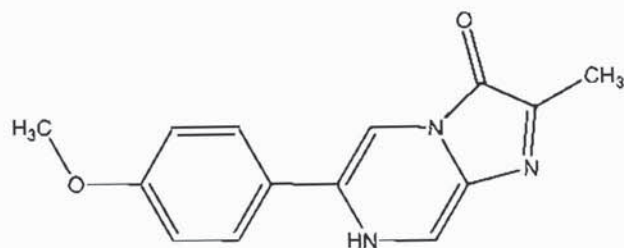
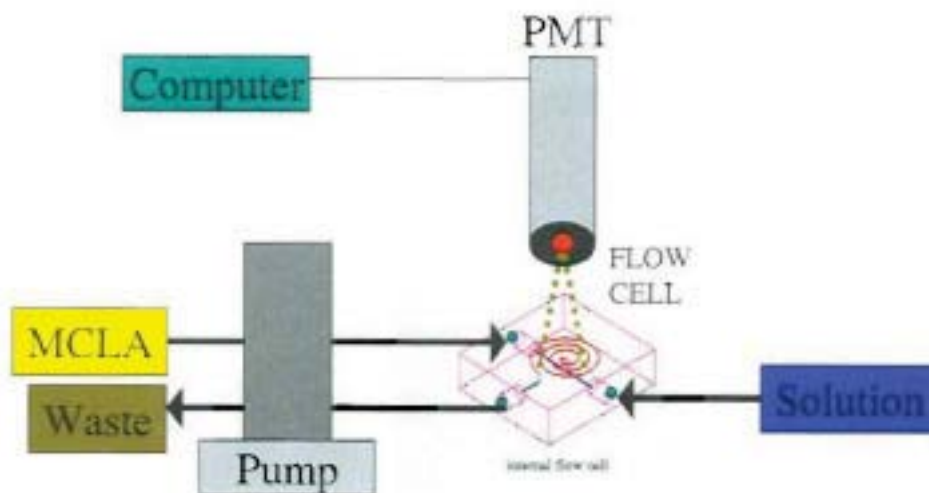


Figure 12. Methylated Cypridina Luciferin Analogue (MCLA)

Using the method described by Rose and Waite, MCLA solution was prepared to a concentration of  $1\mu\text{M}$  and a pH of 6.0. Into 85mL of Milli-Q water, 5mg of MCLA (Molecular Probes) was dissolved in 85ml of water. The solution was then separated into 17-5ml portions which were frozen in 7ml plastic vials at  $-80^{\circ}\text{C}$ . When a new stock of MCLA was required a 5ml portion was thawed and added to Milli-Q water along with 4.1g of sodium acetate and brought to a volume of 1L and titrated to a pH of 6.0 using concentrated HCl. This solution was refrigerated at  $4^{\circ}\text{C}$  in between uses.

### *Chemiluminescence Analysis and Decay Rates*

The Flow Injection Analysis System designed by Boland was set-up for continuous analysis of superoxide. The system (Figure 13) utilized a Rainin Dynamax RP-1 Peristaltic Pump to deliver the superoxide containing sample and the MCLA reagent to a spiral chemiluminescence flow cell, where the two mixed to chemiluminesce.



*Figure 13.* Superoxide Detection system. A peristaltic pump pushes MCLA reagent into a spiral flow cell while pulling a greater volume of waste from the cell waste. This flow rate differential pulls the sample solution into the spiral flow cell. The absence of a pump for the sample solution allows for a shortened flow length and less decay of the sample. In the spiral flow cell the MCLA and the superoxide containing sample solution mix to give off light that is measured by the photomultiplier tube which is calibrated against known superoxide standards.

The resulting signal was measured using a Hamamatsu HC135 photon counting photomultiplier tube, PMT. There was an 8.5 second delay between the time when the solution entered the tube and a resulting signal was recorded. Data acquisition was controlled using Waterville Analytical Software developed with LabView.<sup>5</sup> A waste line carried products out to a bottle, in which the pH was measured to maintain quality control of the MCLA calibration.

Study samples were drawn from a solution through Teflon tubes by the suction created by pumping in the MCLA and pumping out the waste from the flow cell. A baseline of the study solution was collected before any superoxide was added to the solution. Once a steady baseline was collected, using the CL system, production of superoxide on the UV/Vis system began. A 30 second baseline was collected using the UV/Vis before photolysis started. After those 30 seconds the superoxide generation solution was photolyzed for 1 minute after which the Hg lamp was shut off and superoxide produced in this system was pipetted into the study solution. It took approximately 6 seconds between the end of photolysis and the addition to the study solution and 8.5 seconds for the study solution to reach the flow cell to produce a CL signal upon mixing with the MCLA.

The subsequent PMT decay data was exported to a spreadsheet where the baseline CL signal was subtracted. Each day a calibration run was performed using phosphate buffer between pH 10 and 11 to get a calibration coefficient for the MCLA reaction with superoxide. The decay of superoxide at pH 10, fit known decay rates and was slow enough that a value for the PMT signal was effectively constant during the 8.5 second lag time between sampling and CL in the flow cell. The ratio of the intercept to the dilution

corrected superoxide concentration obtained from the spectrophotometric data was used as the calibration coefficient for determining concentrations from the PMT signal.

For the study solutions the baseline subtracted PMT signal was converted to a concentration of superoxide using the calibration coefficient:

$$[O_2^{\bullet-}]_t = \frac{(\text{signal} - \text{baseline})}{C_{\text{pmt}}} \quad (45)$$

The decay of superoxide in natural waters was described above by equation (10).

The loss of superoxide can thus be expressed as the sum of both the first order and second order decay processes:

$$dO_2^{\bullet-} = k_x [O_2^{\bullet-}] dt + k_d [O_2^{\bullet-}]^2 dt \quad (46)$$

The superoxide concentration at time  $t$  can be calculated if the first order and second order decay rates are known. Equation (46) was fit to superoxide decay data using a non linear curve fitting routine, Solver in Microsoft Excel. In practice the second order decay rate is known by equation (6), and this computed value is used to solve equation (46) in terms of  $k_x$  alone.

### *Kinetics Experiments*

Tris-hydroxymethylaminoethane, Tris, was used as a buffer in control experiments. Tris has been shown to have no reaction with superoxide.<sup>17</sup> The Tris buffered experiments were bubbled with nitrogen gas to decrease the natural  $CO_2$  in the solution. In the carbon dioxide experiments, bicarbonate was used to buffer the solution to fix pH and the carbonate alkalinity. Buffer concentrations ranged from 0.1M – 0.001M although 0.1M buffers provided more reproducible results. The solutions were bubbled with gas for at least 15 minutes before the kinetic runs.

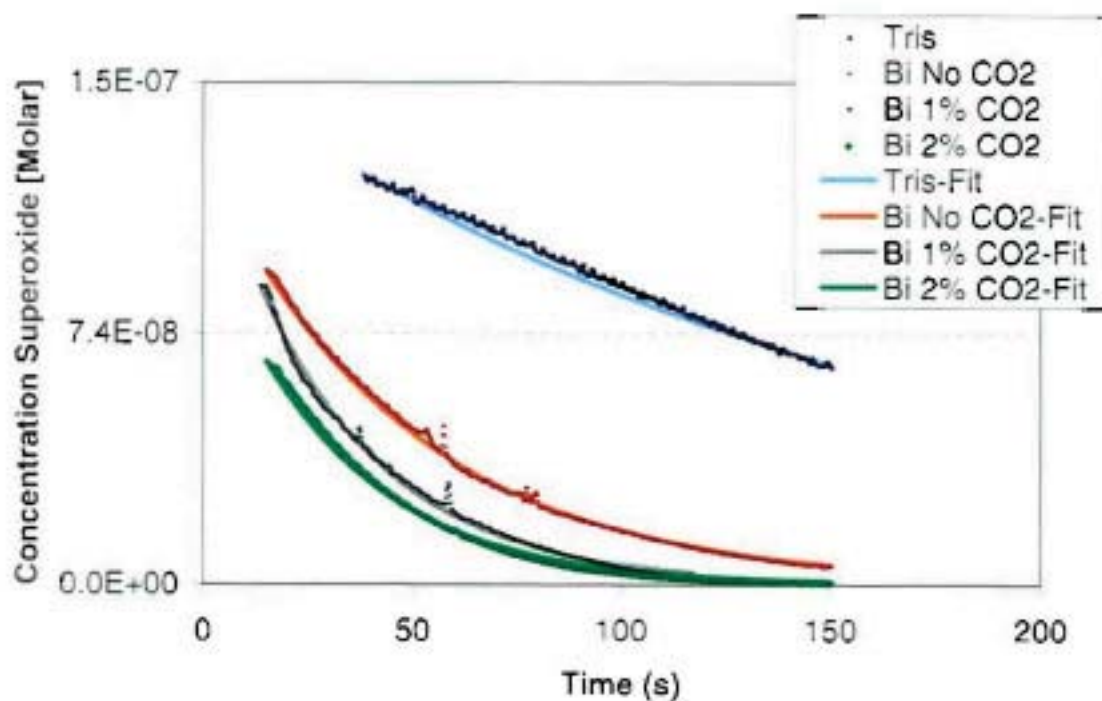


## Results and Discussions

Figure 14 shows comparative data for the decays of superoxide in solution. As expected with the addition of carbonate and carbon dioxide to the system causes the decay of superoxide to become faster. The change in pH is not enough to account for this change. Table 2 shows the first order rate constants calculated for each system, with Figure 15 displaying these results graphically. All these superoxide decay curves may be fit using simple first order decay kinetics. This implies that  $k_x \gg k_d$  for the entire set of experiments.

The Tris buffered solutions unexpectedly showed only first order decay rates. This could be a result of many things. Despite the use of non-carbonate buffers and trying to purge sample and reagent water pure of  $\text{CO}_2$  it is possible that carbon dioxide entered the system. We can observe purely second order decay in the spectrophotometric system where the superoxide concentration is high. When the superoxide concentration is low, as in our study solutions, there is a relatively higher amount of  $\text{H}_2\text{CO}_3^*$ , even in the solutions that were made to be  $\text{CO}_2$  free. There also may be other impurities in the system causing first order decay. Seeing such impurities in the Tris reference solution would also suggest that part of the first order rates in the reactions with carbon dioxide are partially due to other impurities like iron, other trace metals or conceivably organic matter, all competing for superoxide as shown in Figure 2.

The 0.1M bicarbonate containing solution to which no  $\text{CO}_2$  was added showed an increased first order rate over the Tris containing solution. This may be a result of a small amount of dissolved  $\text{CO}_{2(\text{aq})}$  or  $\text{H}_2\text{CO}_{3(\text{aq})}^*$  formed from the bicarbonate coming to equilibrium with the rest of the carbonate system.



*Figure 14.* Decays of superoxide in various systems and curve fits. Extrapolation back to time zero gives different values because initial superoxide concentration varied as a result of the photolysis method. With added carbonate the decay rate increases. With the addition of carbon dioxide the decay becomes even more rapid. Although the pH does decrease from 8.9 for the Tris, to 8.6 for the Bicarbonate without CO<sub>2</sub>, to 8.5 for the 1% solution and 8.3 for the 2% solution, this is not enough to explain the downward shift of the curves.

Table 2. Decay rates as a function of various conditions. The  $[H_2CO_3^*]$  was determined from pH and [Alk], and the  $pCO_2$  was determined from the % $CO_2$  or was assumed to be less than the atmospheric saturation value when  $N_2$  was being bubbled through. The second order rate constants,  $k_d$ , are calculated values determined from equation (6). The first order rate is  $k_x$ . When the fit was made for each of the solutions the second order rate was found to be insignificant. All decays may be fit with simple first order kinetics. The  $[H_2CO_3^*]$  is a combination of both  $H_2CO_{3(aq)}$  and  $CO_{2(aq)}$  and due to the kinetically slow equilibrium it is impossible to determine the exact amount of each.

Buffer / Gas	pH	[TA]	$[H_2CO_3^*]$	$pCO_2$	$\log(k_x)$	$k_x \text{ s}^{-1}$	$\log k_d \text{ M}^{-1} \text{ s}^{-1}$
Tris / $N_2$	8.9		$0.0 < 3.55 \times 10^{-4}$		-2.3	$5.2 \times 10^{-3}$	3.9
Tris / $N_2$	8.5		$0.0 < 3.55 \times 10^{-4}$		-2.0	$9.7 \times 10^{-3}$	4.3
$HCO_3^-$ / $N_2$	8.9	0.1	$2.7 \times 10^{-4} < 3.55 \times 10^{-4}$		-1.9	$1.4 \times 10^{-2}$	3.9
$HCO_3^-$ / $N_2$	8.6	0.1	$5.5 \times 10^{-4} < 3.55 \times 10^{-4}$		-1.7	$2.1 \times 10^{-2}$	4.2
$HCO_3^-$ / 1% $CO_2$	8.5	0.1	$7.0 \times 10^{-4}$	$1 \times 10^{-2}$	-1.5	$3.1 \times 10^{-2}$	4.3
$HCO_3^-$ / 2% $CO_2$	8.4	0.1	$7.8 \times 10^{-4}$	$2 \times 10^{-2}$	-1.4	$3.8 \times 10^{-2}$	4.4
$HCO_3^-$ / 2% $CO_2$	8.3	0.1	$1.1 \times 10^{-3}$	$2 \times 10^{-2}$	-1.5	$3.1 \times 10^{-2}$	4.3

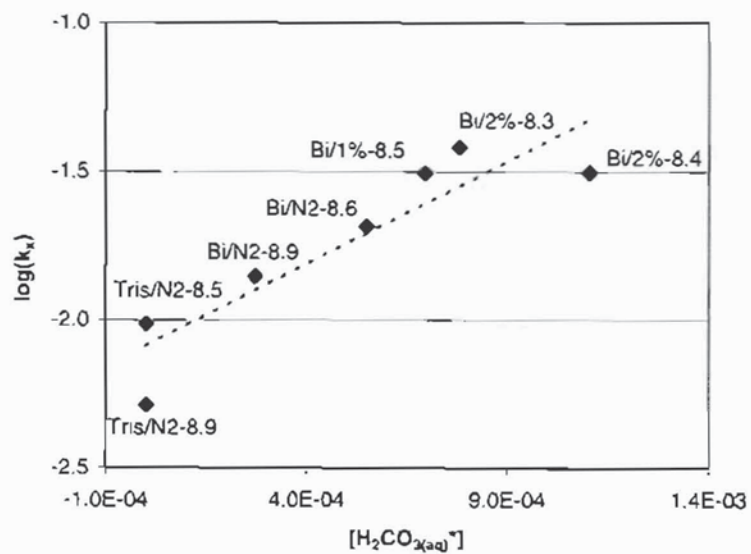


Figure 15. Plot of the log of first order decay rates of superoxide as a function of  $[H_2CO_{3(aq)}^*]$ . Points are labeled by buffer(Tris or Bicarbonate) / Gas (N<sub>2</sub> or %CO<sub>2</sub>) – pH

When  $\text{CO}_2$  is bubbled through the solution the decay rates noticeably increase. Adding would increase both the  $\text{CO}_{2(\text{aq})}$  and the  $\text{H}_2\text{CO}_3^*$  and implies that one of these two species is reacting with the superoxide and not bicarbonate. In these experiments the bicarbonate concentration is relatively constant. However, the dissolved carbon dioxide and carbonic acid concentrations are much larger in the  $\text{CO}_2$  addition experiments, suggesting that an increased rate is dependant on an increased amount of one of these two species. The effect of carbon dioxide on superoxide is more strikingly portrayed when the first order rates are plotted against  $[\text{H}_2\text{CO}_3^*]$  showing a clear increase in rate with increasing  $\text{H}_2\text{CO}_3^*$ . This suggests that there is a relationship between the first order decay rate observed in these experiments and  $[\text{H}_2\text{CO}_3^*]$  or something proportional to  $[\text{H}_2\text{CO}_3^*]$  which would include both  $\text{H}_2\text{CO}_{3(\text{aq})}$  and  $\text{CO}_{2(\text{aq})}$ . Figure 16 contains data from this experiment added to Figure 2. It shows that the carbon dioxide mediated decay is faster than the 100nm scaled disproportionation reaction. It also shows that the carbon dioxide decay lies within the decay range of  $k_x$  and the known aprotic decay of superoxide by carbon dioxide. The difference between sink  $x$  and the experimental rates might be attributed to both the increased amount of carbon dioxide as compared to natural systems and the presence of some impurities.

In addition to the data listed in Table 2 several different experiments were tried that did not provide conclusive results. These experiments consisted of lowering the pH to 3, increasing buffer concentrations, and increasing DTPA concentrations. Lowering the pH allowed for an analysis of a system that was predominately either dissolved carbon dioxide or carbonic acid, with no carbonate or bicarbonate present. The problem was that at this pH the disproportionation reaction was nearly as fast as second order

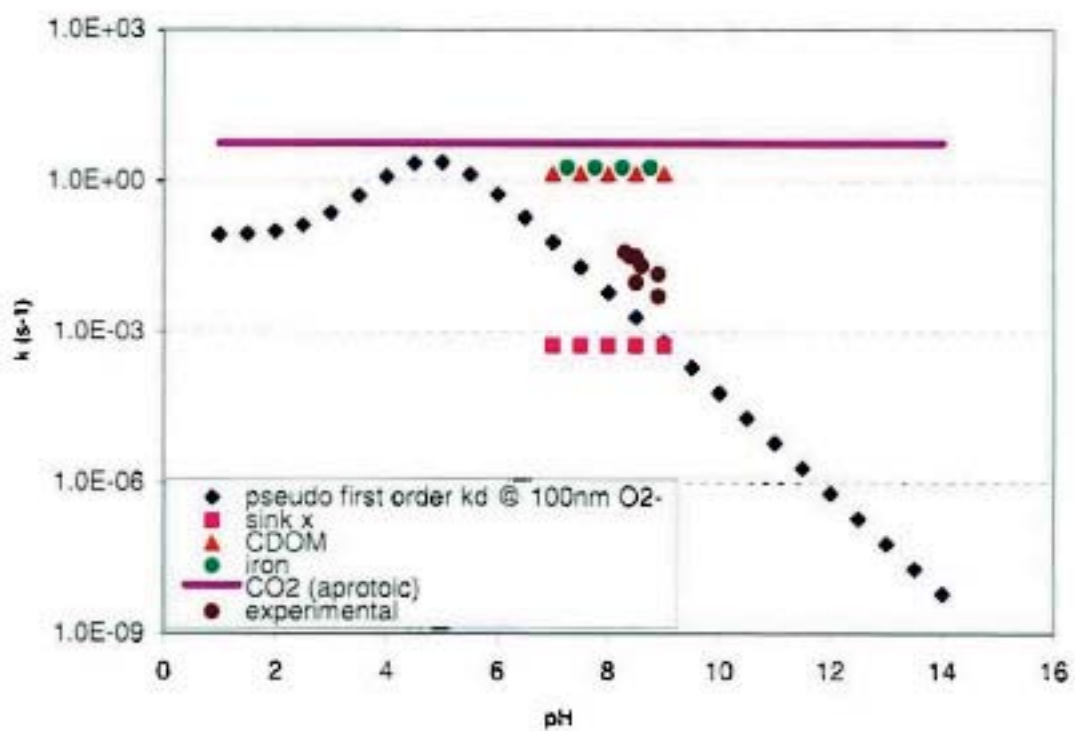


Figure 16. First order decay rates of superoxide with data from this experiment.

Decay and also that DTPA became ineffective increasing the potential for metal contamination. Therefore at low pH and the presence of metals controlled the decay.

Increasing DTPA concentrations at higher pH's helped to improve the reproducibility of the results but at a high enough concentrations DTPA became its own buffer and started affecting the pH of the system, making it harder to control the pH using carbon dioxide. Increasing the other buffer concentrations also worked in generating more reproducible results but also resulted in a longer time for equilibrium to be established. In the final set of experiments buffer concentrations of 0.1 M and DTPA concentrations of  $10^{-3}$  M were used and produced the best set of results.

Still there are concerns about impurities affecting the decay rate as well as several alternative mechanistic pathways for the carbon dioxide-superoxide sink resulting in both first and second order rates. Possible contaminants could come from reagents even the DTPA. Simple dust and dirt in the lab may also play a role. The pH probe reference solution travels across the pH probe membrane into the solution and may carry with it possible contaminants. Future work must be very concerned with metal contamination of all solutions.

## Conclusions

In aqueous systems dissolved carbon dioxide, either in the  $\text{CO}_{2(\text{aq})}$  or the  $\text{H}_2\text{CO}_{3(\text{aq})}$  forms maybe responsible for a first order decay of superoxide. Bubbling carbon dioxide gas through carbonate system made the first order decay rate increase substantially. Interpreting the rate constants established in Table 2 in terms of specific species in solution is less straight forward. Not all data fit a linear relationship as shown in Figure 15 and the Tris experiments showed signs of contamination. Therefore we have exciting evidence that  $\text{CO}_2$  does indeed increase the decay rate of superoxide. However, the data is not robust enough to allow a specific mechanism for  $\text{CO}_2$  mediated decay of superoxide to be proposed.

## Future Work

This work was a first step. Future work reproducing Bielski's pure second order decay data for the disproportionation reaction of superoxide at nanomolar concentrations in a solution free of  $\text{CO}_2$  needs to be done. This control experiment will give us a zero point that would ensure that the solution is free of impurities that would affect both first order and second order rates. I feel that Tris buffer, because of its pKa and proven non-reactivity with superoxide is the best medium for a control.

Simultaneous analysis of hydrogen peroxide production using an acridinium ester based chemiluminescence system was employed to measure the production of hydrogen peroxide from the disproportionation reaction. A purely second order decay because of reactions (4) and (5) would result in half the amount of hydrogen peroxide



being produced as a result of superoxide decay. A simultaneous analysis should show this increase. In preliminary experiments a small unquantifiable increase was noted above an elevated baseline present from gas exchange with lab air. If enough natural hydrogen peroxide can be scrubbed out of all the water used in this experiment, and if the flow tube is short then it should be possible to notice the production of hydrogen peroxide.

The use of superoxide creating enzymes can be employed as a source to create a steady state concentration of superoxide. Carbon dioxide can be added to a system containing xanthine/xanthine oxidase and changes in the steady state concentration of superoxide measured. The addition of just carbon dioxide gas would be expected to slowly decrease the steady state concentration of superoxide.

Another approach best step would be to increase the superoxide concentration to micro molar levels. The larger concentration would show less effects of contaminants because the contaminants will be a relatively smaller sink compared to the second order decay at these concentrations.

Its also possible that CO<sub>2</sub> may cause second order decay of superoxide. Consider reactions (17) and (18). If equation (17) is fast and reversible then the equilibrium is defined by

$$K_x = \frac{[CO_4^{\bullet-}]}{[CO_2][O_2^{\bullet-}]} \quad (47)$$

Assuming reaction (18) is slow then we can define the rate limiting expression:

$$\frac{dO_2^{\bullet-}}{dt} = \frac{dCO_4^{\bullet-}}{dt} = k_2[O_2^{\bullet-}][CO_4^{\bullet-}] \quad (48)$$

Combining (47) and (48) gives a second order rate expression

$$\frac{dO_2^{\bullet-}}{dt} = (K_1 k_2 [CO_2]) [O_2^{\bullet-}]^2$$

This effect could be very difficult to observe, considering that the first order carbon dioxide decay is much greater than the disproportionation second order decay. It might be possible to quantify this reaction as an acceleration of the second order rates as elevated superoxide and CO<sub>2</sub> concentrations.

It will also be necessary to work out the response of the carbonate system to the presence of high amounts of DTPA used for contaminant removal. There is also a need to ensure that the carbon dioxide gas exchange system can come to equilibrium. Care must also be taken in creating cleaner experiments. Clearly, controlling contaminations is the first priority for future work.

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