

OXIDATION OF BIOLOGICAL MOLECULES WITH BICARBONATE-  
ACTIVATED HYDROGEN PEROXIDE AND THE DECOMPOSITION OF  
HYDROGEN PEROXIDE CATALYZED BY MANGANESE(II) AND  
BICARBONATE

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL  
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT  
OF THE REQUIREMENTS FOR THE DEGREE OF  
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2004

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Michael S. Mitchell

This document is dedicated to my fiancé and my family for all their help and support during the last few years.

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May 2004

Chair: David E. Richardson  
Major Department: Chemistry

Most reactions involving hydrogen peroxide use catalysts. Manganese(II) can catalyze both oxidation reactions involving bicarbonate-activated hydrogen peroxide and the disproportionation of hydrogen peroxide in the presence of bicarbonate. The oxidation of biological molecules, such as guanosine monophosphate and amino acids, with bicarbonate-activated hydrogen peroxide is accelerated by addition of manganese(II). In the oxidation of the amino acids, the peptide is oxidized by two consecutive reactions, deamination followed by decarboxylation. Buffers have been shown to affect the rate, and bicarbonate/carbon dioxide and phosphate buffer were used to control pH.

To obtain information of the mechanism of the oxidation reactions, decomposition of hydrogen peroxide in the presence of bicarbonate and manganese(II) has been examined. Certain buffers, such as phosphate and HEPES, reduce the rate of the

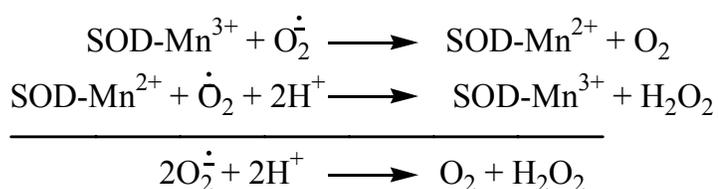
manganese(II)-catalyzed decomposition of hydrogen peroxide. No effect on the rate of the decomposition of hydrogen peroxide has been detected with the borate buffer. Using the borate buffer, the reactant orders for bicarbonate (second order at  $[\text{HCO}_3^-] < 0.4 \text{ M}$ ) and manganese(II) (first order at  $[\text{Mn}^{2+}] < 5 \text{ }\mu\text{M}$ ) have been determined for the catalyzed peroxide disproportionation. The decomposition also showed no ionic strength effect. The fate of the metal is still being examined.

## CHAPTER 1 INTRODUCTION

Hydrogen peroxide and other *reactive oxygen species* (ROS) oxidize biological molecules, resulting in the cellular oxidize stress.<sup>1</sup> In biological conditions, oxidation reactions with hydrogen peroxide usually proceed slowly in the absence of a catalyst. Manganese(II) can catalyze two reactions involving hydrogen peroxide in the presence of bicarbonate. The decomposition of hydrogen peroxide and the oxidation reactions involving hydrogen peroxide are both catalyzed by manganese(II)-bicarbonate mixtures.<sup>2;3</sup> Peroxymonocarbonate, a possibly biologically relevant ROS, forms by the reaction of peroxide and bicarbonate.<sup>4;5</sup> The study presented here examines both the decomposition of hydrogen peroxide and the oxidation reactions of biological molecules by hydrogen peroxide catalyzed by manganese(II) and bicarbonate.

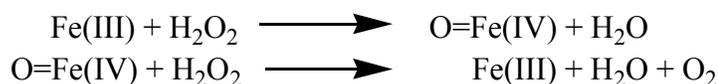
**Reactive oxygen species.** ROS are oxygen-containing molecules capable of oxidizing several classes of molecules. Superoxide radical anion, hydroxyl radical anion, and hydrogen peroxide are examples of biologically relevant ROS molecules.<sup>1</sup> These ROS molecules may oxidize biological molecules *in situ*, increasing the oxidative stress on cells. For example, the oxidation of an active site of an enzyme by hydrogen peroxide can cause a decrease in its activity.<sup>6;7</sup> However, certain enzymes also use the oxidation potential of hydrogen peroxide to oxidize toxins, such as hydrocarbons. The solubility of the resulting product is higher than that of the original hydrocarbon, so the oxidation makes the removal of the toxin easier.

In cells, the formation of hydrogen peroxide primarily occurs in mitochondria. Molecular oxygen is the initial reactant for this pathway. Oxygen is used in the mitochondria for energy formation. In the normal process of oxidative phosphorylation, oxygen gains four electrons, resulting in the formation of adenosine triphosphate and water. However, incomplete reduction of oxygen occurs in a small fraction of oxygen reduction. This pathway results in the formation of other ROS. Initially, the pathway for the formation of hydrogen peroxide involves a one-electron reduction of molecular oxygen, forming a superoxide radical anion. The enzyme superoxide dismutase (SOD) decomposes the superoxide radical anion into molecular oxygen and hydrogen peroxide [Scheme 1-1].



Scheme 1-1: These equations show an enzymatic pathway for the formation of hydrogen peroxide by Manganese-SOD.

The SOD is an example of pathways for elimination of ROS from cells. Many families of SOD are known, and they use different metals at the active site. These metals include manganese and iron. For the removal of hydrogen peroxide, cells use a different enzyme, catalase. This enzyme uses iron to decomposes hydrogen peroxide into molecular oxygen and water [Scheme 1-2].



Scheme 1-2: The two half reaction to eliminate hydrogen peroxide in cells.

**Catalytic decomposition of hydrogen peroxide.** Catalysts are usually required for oxidations by or decomposition of hydrogen peroxide due to its relatively low reactivity.

The function of a catalyst in any reaction is to reduce activation energy by one of two different means. The first is to increase the energy level of the starting materials. The second is to lower the energy requirements of the transition state.

Previous studies examined the catalytic ability of other transition metals to decompose hydrogen peroxide as well.<sup>8</sup> Manganese and copper have been studied in detail. One study examined the rate of reaction at a variety of pH levels. There was a decrease in the rate of reaction as pH levels increased with all of the transition metals studied. The decrease in rate at high pH was due to the formation of insoluble metal hydroxides. Different metal complexes were used to increase solubility and the rate of decomposition.

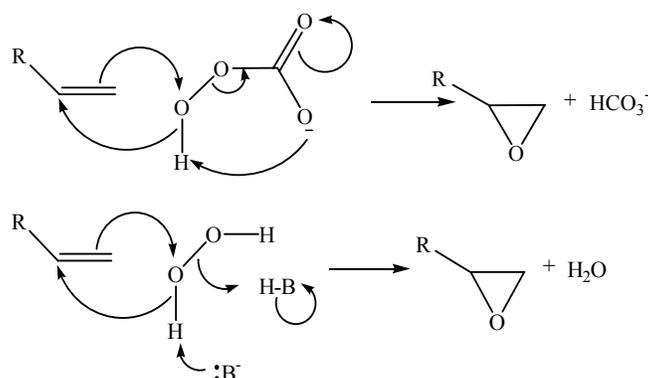
A variation of the rate of reaction was obtained from decomposing hydrogen peroxide studies with different transition metal complexes, such as with manganese(II). The data demonstrate that as the environment around the metal changes, the rate of reaction also changes. Many manganese(II) complexes have been studied, including  $\text{Mn}(\text{phen})_2^{2+}$ ,  $\text{Mn}(\text{dipy})_2^{2+}$ ,  $\text{Mn}(\text{acac})_2^{2+}$ ,  $\text{Mn}(\text{EDTA})_2^{2+}$ ,  $\text{Mn}(\text{his})_2^{2+}$ , and  $\text{Mn}(\text{TETA})_2^{2+}$  (ranked in order of increasing activity).<sup>9</sup> For the reactions presented here, the formation and effects of different metal complexes will be further examined in Chapter 2.

**Oxidation reactions by hydrogen peroxide.** The Richardson group have reported experiments examining hydrogen peroxide oxidation reactions. One study demonstrated sulfide oxidation by hydrogen peroxide.<sup>5</sup> Methionine was also studied at the same time.<sup>10</sup> Sulfides are oxidized to sulfoxide and sulfones. The oxidation of the protein  $\alpha 1$ -PI was investigated. When this protein and hydrogen peroxide were mixed, a decrease in its activity resulted. This was due to the oxidation of a crucial methionine in the  $\alpha 1$ -PI



Further studies compared the oxidation of alkenes with peroxymonocarbonate/hydrogen peroxide. While both peroxymonocarbonate and hydrogen peroxide oxidize some alkenes to epoxides, the rate of oxidation by peroxymonocarbonate is greater.

As depicted in Scheme 1-4, one difference in the oxidations was the leaving group. Bicarbonate is the leaving group after the oxidation with peroxymonocarbonate, and water is the leaving group after the oxidation with hydrogen peroxide. A lower pKa value may imply greater product stability, in turn, increasing the leaving group tendency. The pKa of bicarbonate is 10.3 while the pKa of water is 15; therefore, using the pKa, one can conclude the peroxymonocarbonate should be more reactive than hydrogen peroxide.<sup>13</sup>



Scheme 1-4: Oxidation of an alkene by both peroxymonocarbonate and hydrogen peroxide.<sup>13</sup>

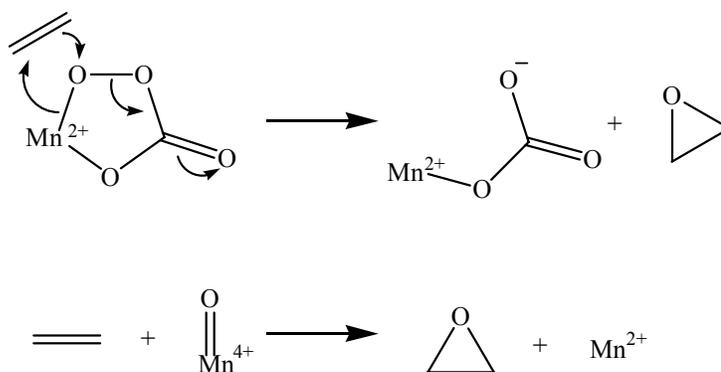
Yao and Richardson also examined oxidations by peroxymonocarbonate in pure water versus mixed solutions (alcohols and water), performed the reactions with micelles, and analyzed the oxidation of different types of substrates.<sup>14;15</sup> One result demonstrated the catalytic ability of transition metals involving the oxidations by peroxymonocarbonate. Manganese(II) has demonstrated the greatest reactivity of all the metal studied.<sup>4;16</sup>

**Chemistry of peroxymonocarbonate/manganese(II) oxidation.** Burgess, Sychev, and Stadtman all lead studies examining the oxidation of different substrates by hydrogen peroxide in the presences of bicarbonate and manganese(II).<sup>16;19-22</sup> Both Sychev and Stadtman suggested the involvement of radicals in the oxidations. However, the proposed mechanisms were slightly different depending on the substrate.

Sychev and coworkers examined the oxidation of different dyes with the addition of radicals. For some dyes, the rates of reaction decreased. They therefore proposed two different mechanisms, an inner-sphere or an outer-sphere mechanism, for the different results. If the rate of reaction decreased, the result supported an outer-sphere mechanism.<sup>22</sup> If detecting no decrease, the proposed pathway was then including an inner-sphere mechanism.<sup>21</sup>

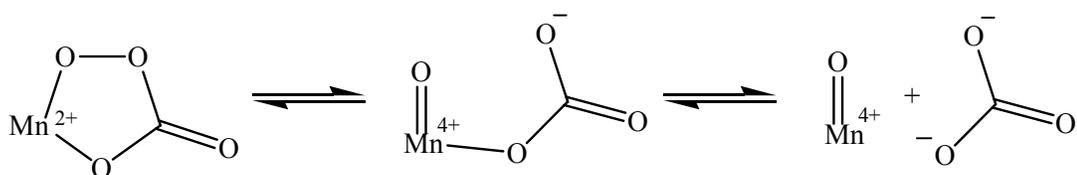
Stadtman's laboratory examined the oxidation of amino acids.<sup>2</sup> No decrease in rate was found with the addition of radical traps.<sup>20</sup> They therefore concluded that the amino acid oxidation mechanism involved a caged radical. Their proposed mechanism is similar to the Sychev's proposed inner-sphere mechanism.

The Burgess group studied of the oxidation of alkenes by peroxymonocarbonate in the presence of manganese(II). Unlike Stadtman and Sychev, Burgess' group studied the reactions in mixed dimethylformamide/water. Knowing about peroxymonocarbonate, they suggested pathways besides an inner-sphere or outer-sphere radical mechanisms [Scheme 1-5]. Another study by Burgess and coworkers examined the oxidation of cis- and trans- alkenes. When performing an oxidation of a cis-alkene in the mixed solvent, the reactions produces a mixture of cis- and trans- epoxide, demonstrating that an intermediate in the reaction can rotate about the original double bond.<sup>4</sup>



Scheme 1-5: Proposed mechanisms by Burgess. The second may proceed two ways, concerted or stepwise.<sup>4</sup>

There are a few hypotheses explaining different metal pathways, as seen in Scheme 1-5. One hypothesis suggests that peroxymonocarbonate binds the metal. Upon binding, peroxymonocarbonate may become a better oxidant. Another hypothesis proposed that peroxymonocarbonate oxidizes manganese(II) to either manganese(III) or manganese(IV), possibly forming a metal oxide [Scheme 1-6]. The metal oxide then acts as the oxidant. Sychev's group detected the presence of a manganese(III) species during their reactions.<sup>9</sup> Burgess' laboratory detected the presence of a manganese(IV) species during their oxidations.<sup>4</sup> The decomposition of hydrogen peroxide in the presence of bicarbonate and manganese(II) is being examined to propose the pathway for the metal oxidation reactions.



Scheme 1-6: Formation of the metal oxide species by peroxymonocarbonate.<sup>4</sup>

### Decomposition of hydrogen peroxide by bicarbonate and manganese(II).

Manganese(II) and bicarbonate catalyze the decomposition of hydrogen peroxide.<sup>2;17</sup>

Therefore, in studying oxidations by peroxymonocarbonate and manganese(II), one must consider the decomposition rate of hydrogen peroxide. In most cases, the oxidation reactions proceed faster than the decomposition. However, the rate of the decomposition reaction is higher when oxidizing electrophilic alkenes to epoxide. Since the addition of metal increases the rate of the decomposition of hydrogen peroxide, there is less peroxymonocarbonate formed in the solution to oxidize the alkene.

In the late 1970s and early 1980s, Sychev et al. studied examining the pathway for the decomposition of hydrogen peroxide in the presence of bicarbonate and manganese(II) by monitoring oxygen production.<sup>18</sup> Sychev and coworkers concluded the rate law order is first order for both manganese(II) and hydrogen peroxide, and the reaction is second order in bicarbonate for the concentration range of 0 to 0.4 M. For concentrations of bicarbonate above 0.4 M, the reaction is zero order in bicarbonate. Additionally, a pH dependency was also quantified, showing an increase in reaction rate in the pH range = 7.0 to 7.6. A rate decrease was detected above 7.6. At pH levels above 7.6, the formation of  $\text{MnCO}_3$ , an insoluble product not active in the decomposition reaction, explained the decrease in rate.

In the early 1990s, Stadtman et al. also studied of the decomposition of hydrogen peroxide in the presence of bicarbonate and manganese(II) by monitoring oxygen production.<sup>19</sup> Similar to Sychev and coworkers results, the reaction is first order in both manganese(II) and hydrogen peroxide. However, the rate order for bicarbonate is third order. Possibly, the rate order changes due to the use of a different buffer by Stadtman and coworkers.

Stadtman used other buffers such as phosphate or 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) to maintain pH. When using these molecules, the reaction rate decreases. The addition of carbon dioxide to the atmosphere above the solution created a buffering effect near physiological pH (7.4).

Stadtman et al. also examined the addition of hydrogen peroxide to a completed reaction.<sup>19</sup> Compared to the initial rate of the reaction, the decomposition rate decreased with the addition of hydrogen peroxide. During the initial reaction, the formation of a nonreactive metal species caused the variation in rate.

**Goals.** The experiments described in this thesis were designed to address the following questions regarding the reactions of bicarbonate-activated peroxide with biological molecules:

1. Can peroxy carbonate oxidize biological molecules?
2. What are the effects (if any) of different buffers?
3. Are the products that Stadtman et al. detected the only ones produced under their conditions?

## CHAPTER 2 RESULTS AND DISCUSSION

**Disproportionation of hydrogen peroxide.** Manganese(II) can catalyze both the decomposition of hydrogen peroxide in the presence of bicarbonate and oxidation reactions by bicarbonate-activated hydrogen peroxide.<sup>2;3</sup> Bennett and Sychev have obtained similar results for both kinds of reactions. The rate orders in bicarbonate and manganese(II) for oxidation and disproportionation are the same in most reports.<sup>16;18</sup> It may be possible to answer some questions regarding the oxidation reactions by studying the simple disproportionation reaction.

The groups of Sychev and Stadtman studied the decomposition of hydrogen peroxide by manganese(II)/bicarbonate.<sup>18;19</sup> The two groups obtained similar results. For both groups, the rate orders for both hydrogen peroxide and manganese(II) were first order. Additionally, the formation of radicals was suggested to occur during the decomposition reaction. However, one difference was the order in bicarbonate (Sychev proposed second order and Stadtman third order). The difference in rate order may have been due to the changes in reaction conditions.

When studying kinetics, a buffer is used to maintain pH. However, the buffer should not normally affect the reaction. According to Stadtman et al., some buffers, such as phosphate, change the rate of hydrogen peroxide decomposition in the presence of bicarbonate and manganese(II).<sup>19</sup> Richardson and coworkers used phosphate buffers

during the oxidation reactions, but they never examined the effect of the buffer on the reactions.

The formation of insoluble metal phosphates is a potential problem when using a phosphate buffer in reactions involving transition metals. To determine if problems may surface from solubility during a reaction, one can use the  $K_{sp}$  value of the salt in question. As the  $K_{sp}$  value decreases, the maximum soluble concentration also decreases. The  $K_{sp}$  of  $MnHPO_4$  is  $3.6 \times 10^{-8}$ . In many of the reactions studied here, 5  $\mu$ M manganese(II) is added to the reaction. To keep most of the metal soluble, the phosphate buffer concentration must be maintained below 0.009 M. In most reactions, the concentration of the buffer is near 0.1 M. Therefore, phosphate decreases the rate of reaction by precipitation of manganese(II). Recently obtained data supports this hypothesis. The rate increases as the concentration of phosphate decreases, as shown in Figure 2-1.

Goode designed families of buffers that have a low affinity for metals while maintaining a pH near that of biological reactions. Because of these characteristics, two buffers were chosen to study the decomposition of hydrogen peroxide, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and 4-(2-Hydroxyethyl)piperazine-1-propanesulfonic acid (EPPS). The initial decomposition rate using HEPES was slower than previous results obtained by Burke, a member of the Richardson group. The change in rate may have been due to the different pH of the solution. A pH profile was determined after examining other properties. The experiments resulted in an increase in rate as the pH increased from 7.0 to 7.9. When the pH reached 8.3, the rate at this pH level was about twelve times slower than the previously measured rate in Richardson's group at the same pH.

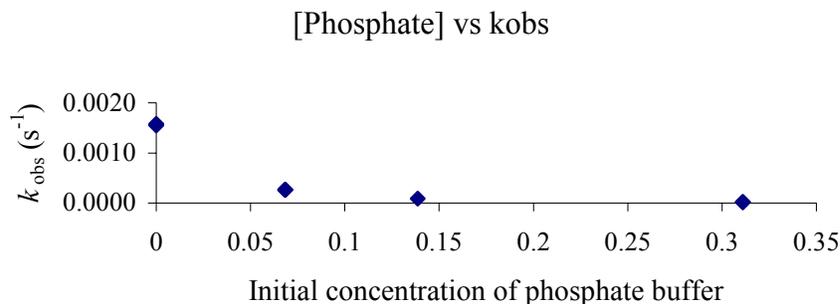


Figure 2-1: Dependence of Phosphate on the disproportionation of hydrogen peroxide. Fit of the Rate Law to the  $k_{1obs}$  for the observed Phosphate Buffer data. The conditions for the reactions involved 0.2 M sodium bicarbonate, 0.1 M hydrogen peroxide, 5  $\mu$ M  $Mn^{2+}$ , 0 – 0.31 M Phosphate Buffer, 0 – 0.5 M sodium acetate. The pH of these reactions was completed at 8.3.

A difference in reaction methods may explain the difference in rate. The change in procedure was not allowing time for the formation of peroxymonocarbonate. However, an unexpected result was determined after mixing bicarbonate and hydrogen peroxide for 20 minutes in the presence of HEPES. HEPES became oxidized. Using Proton Nuclear Magnetic Resonance ( $^1H$ -NMR), the oxidation of both tertiary amines in the HEPES structure was detected (Figure 2-2). The oxidation of tertiary amines by peroxymonocarbonate, which requires no metal, has been detected before by members of the Richardson group. However, the oxidation does not necessarily explain the decrease in rate. A possible hypothesis is that the metal and HEPES form an insoluble complex. If this insoluble complex forms, the profile obtained for the observed rate versus the concentration of HEPES should be similar to the profile of phosphate buffer. Currently, this hypothesis is being examined. Stadtman also saw a decrease in rate during the oxidation by hydrogen peroxide in the presence of HEPES.<sup>19</sup> This result is another similarity that exists between the oxidation reactions and the decomposition reactions.

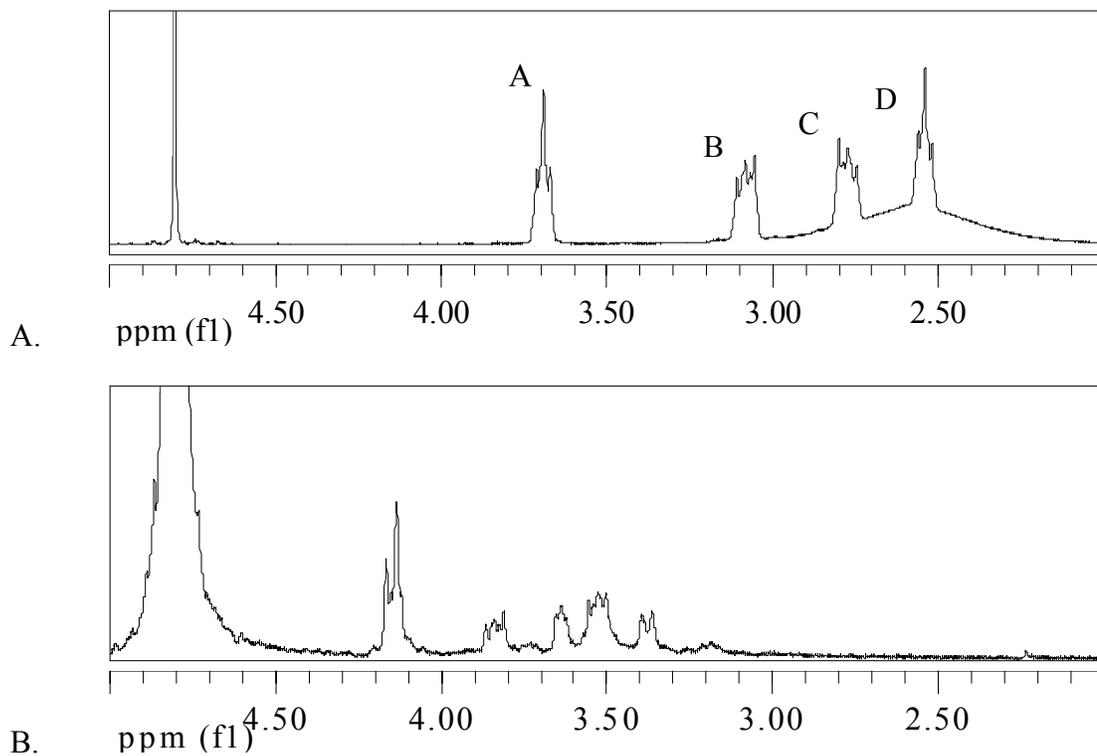
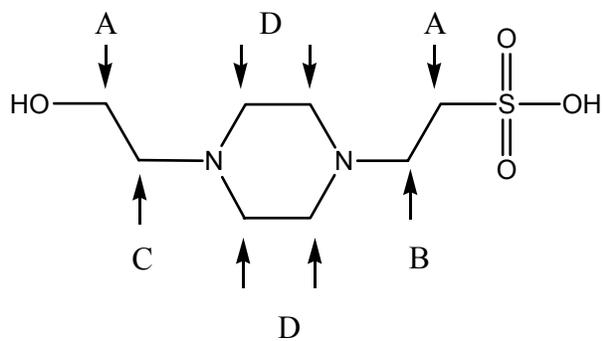


Figure 2-2: Oxidation of HEPES. (A) NMR spectrum of HEPES, buffered in 0.5 M phosphate buffer near pH of 8.3. (B) Oxidation of HEPES. The reaction consisted of 0.1 M HEPES, 0.15 M hydrogen peroxide, and .23 M bicarbonate, buffered in 0.5 M phosphate buffer near pH of 8.3.

Boric acid/borate was also used as a buffer and the pH of the solution was near 8.3.

A reaction buffered with borate was compared to a reaction that contained sodium acetate (both reactions were maintained near 8.3). The two rate constants were nearly identical.

To further examine if the buffer interferes with the oxidation, the rate order in the buffer was examined, and it was zero order (Figure 2-3).

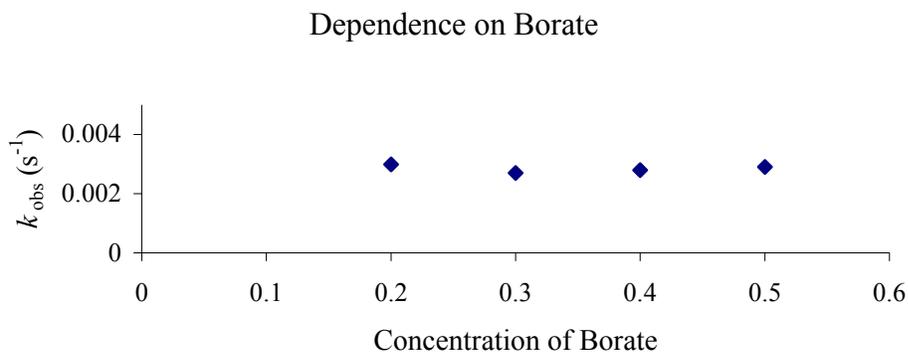


Figure 2-3: Dependence of Borate. In a three-milliliter solution, 0.4 M sodium bicarbonate, 3  $\mu\text{M}$  manganese(II) sulfate, and 0.2 – 0.5 M borate buffer were mixed. The reactions were started by adding 25  $\mu\text{L}$  of 30% hydrogen peroxide and monitored using the UV-Vis at 264 nm. Trace metal was removed from sodium bicarbonate and borate buffer by Chelex.

Following the buffer dependence, different background reactions were examined.

Buffered at 8.3, the background reactions consisted of the following:

1. Borate buffer system with hydrogen peroxide and manganese(II)
2. Borate buffer system with hydrogen peroxide<sup>4</sup>
3. Borate buffer system with hydrogen peroxide and bicarbonate
4. Manganese(II) and bicarbonate
5. Manganese(II) and hydrogen peroxide

During these reactions, no disproportionation was detected throughout the duration of a reaction with all the components. Over a long period, some peroxide decomposition occurred with the reactions containing bicarbonate and hydrogen peroxide, since peracids—e.g. hydrogen peroxide and peroxymonocarbonate—decompose other peracids. Trace metals from the sodium hydroxide are a possibility to explain the slightly accelerated decomposition.

Next, the ionic strength effect was examined by increasing the concentration of acetate in solutions containing 4  $\mu\text{M}$  manganese(II). The results showed no change in rate with variation in ionic strength, as depicted in Figure 2-4. During an oxidation study of 4-vinylbenzenesulfonic acid, the same result was obtained.<sup>16</sup>

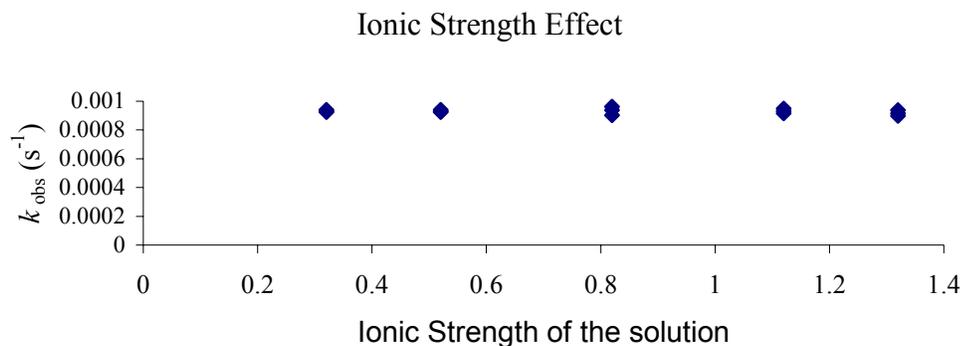


Figure 2-4: Fit of the Rate Law to the  $k_{\text{obs}}$  for the ionic strength data. The conditions for the reactions involved 0.2 M sodium bicarbonate, 0.1 M hydrogen peroxide, 5  $\mu\text{M}$   $\text{Mn}^{2+}$ , 0.1 – 0.7 M sodium acetate, and 0.1 – 0.6 M sodium perchlorate. The reaction was buffered in borate at 8.34.

Next, the rate orders of bicarbonate [Figure 2-5a] and manganese(II) [Figure 2-5c] were determined. Similar to Sychev's results,<sup>18</sup> the rate order of bicarbonate from 0 to 0.4 M is second order [Figure 2-5b]. When the concentration of bicarbonate was between 0.4 and 0.5 M, the dependence decreased to a zero order. The rate decreased above 0.5 M bicarbonate, likely due to the formation of insoluble  $\text{MnCO}_3$  or  $\text{MnHPO}_4$ . The formation of  $\text{MnHPO}_4$  may be due to phosphates being a contaminant in sodium bicarbonate and hydrogen peroxide. The  $K_{\text{sp}}$  of  $\text{MnCO}_3$  is  $3.19 \times 10^{-7}$ .

Following the bicarbonate dependence, the rate order of manganese(II) was obtained. The results were similar to the results previously obtained by Richardson's group for the lower concentrations of manganese(II). From 1 - 5  $\mu\text{M}$  manganese(II), the results showed a first order dependency. Above 5  $\mu\text{M}$ , the rate slowly decreased. The start of a zero order dependency was detected near 40  $\mu\text{M}$ . The decrease may be due to the formation of the insoluble  $\text{MnHPO}_4$  or  $\text{MnCO}_3$ . Phosphates are a contaminant in hydroxide. After removing the metal contaminants from the buffer, phosphates are still in solution, and they can effect the rate.

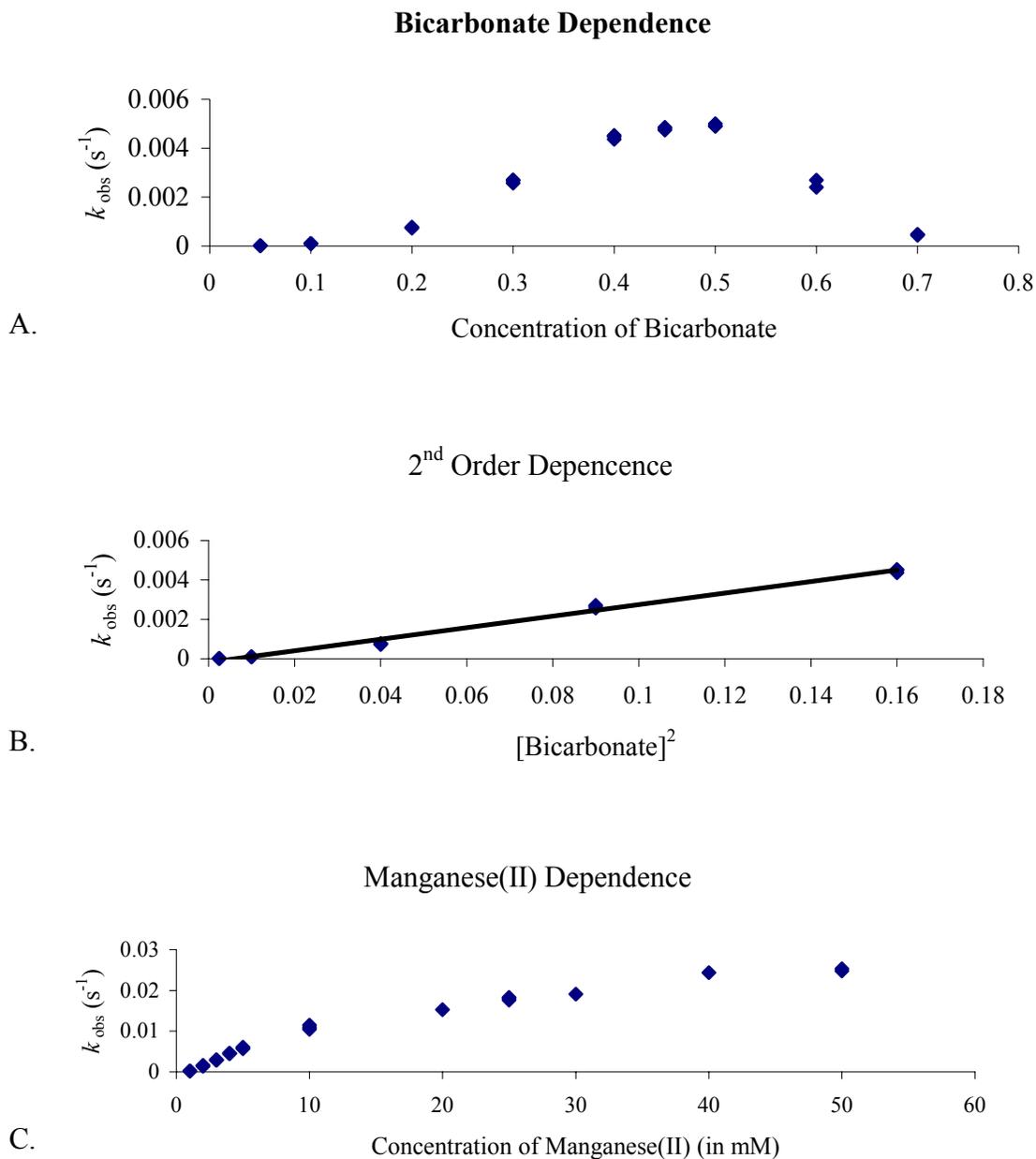


Figure 2-5: Dependence of bicarbonate and manganese(II). (A): Fit of the rate law to the  $k_{1\text{obs}}$  for the observed bicarbonate data. The conditions for the reactions involved 0.05 – 0.7 M sodium bicarbonate, 0.1 M hydrogen peroxide, 4  $\mu\text{M}$   $\text{Mn}^{2+}$ , and 0.5 M borate buffer. The reaction was buffered in borate at 8.34. (B) Data from A graphed as  $k_{\text{obs}}$  vs.  $[\text{bicarbonate}]^2$ , (C): Fit of the Rate Law to the  $k_{1\text{obs}}$  for the observed manganese(II) data. The conditions for the reactions involved 0.4 M sodium bicarbonate, 0.1 M hydrogen peroxide, 1 – 50  $\mu\text{M}$   $\text{Mn}^{2+}$ , 0.5 M borate buffer. The reaction was buffered in borate at 8.34.

The reaction rates were similar to the data obtained previously by Richardson's group for the manganese(II) concentrations below 4  $\mu\text{M}$ . In the current study, the obtained reaction rates above 4  $\mu\text{M}$  manganese(II) were greater than the value obtained previously. Another difference in the methods was the addition of acetate in the previous study to help maintain pH near 8.3. Stadtman also reported a decrease in rate with acetate in solution, but previously, Richardson's group detected no change in reaction rate with the addition of acetate. One possibility for the decrease in rate is the formation of a soluble manganese(II)-acetate dimer. The metal may have fewer coordination sites, resulting in a decrease in rate.

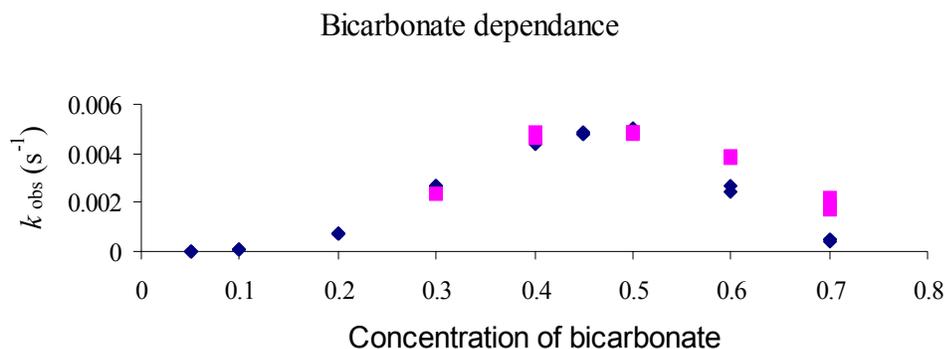
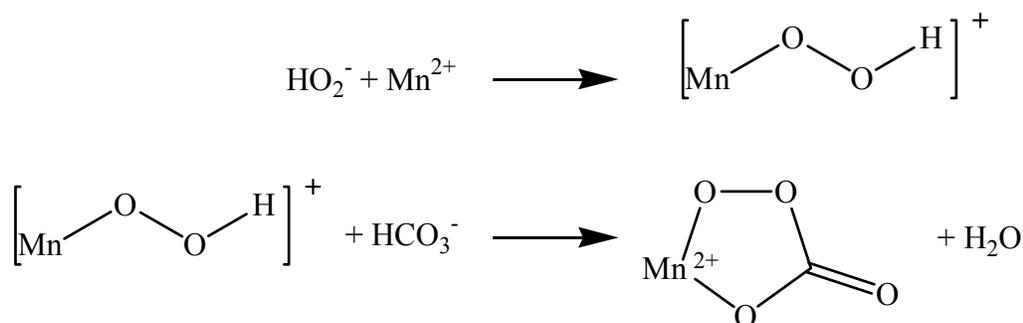


Figure 2-6: Comparison of the rate orders of reactions where peroxymonocarbonate was allotted time to form (squares) compared to reactions that did not (diamonds). Fit of the Rate Law to the  $k_{1\text{obs}}$  for the observed bicarbonate data. The conditions for the reactions involved 0.05 – 0.7 M sodium bicarbonate, 0.1 M hydrogen peroxide, 4  $\mu\text{M}$   $\text{Mn}^{2+}$ , and 0.5 M borate buffer. The reaction was buffered in borate at 8.34.

After obtaining the rate orders of bicarbonate and manganese(II), other properties of the reaction were started to be studied by some researchers in Richardson's group. One hypothesis states that the hydrogen peroxide decomposition involves peroxymonocarbonate. A comparison between two reactions was completed. One

reaction allowed time for peroxymonocarbonate to form by mixing bicarbonate and hydrogen peroxide for 20 minutes without metal present. The other reaction reacted all the components immediately [Figure 2-6]. Possibly due to the formation time for peroxymonocarbonate, no change in rate with the lower concentrations of bicarbonate was detected. For the reactions where bicarbonate and hydrogen peroxide were mixed without metal, an increase in rate was found for the higher concentrations of bicarbonate. However, the rate detected was lower than the rate obtained at 0.4 M bicarbonate. At 0.7 M of bicarbonate, the equilibrium concentration of peroxymonocarbonate is 0.006 M. Through the formation of peroxymonocarbonate, there would be less carbonate in solution. When the carbonate concentration decreases, the soluble concentration of metal would increase. The effect would be an increased rate of reaction. The result does not eliminate the possibility that peroxymonocarbonate has a role in the decomposition of hydrogen peroxide. Another hypothesis states manganese catalyzes the formation of peroxymonocarbonate.<sup>4</sup> Burgess and coworkers have proposed a mechanism for such a reaction [Scheme 2-1].



Scheme 2-1: The proposed mechanism of how manganese(II) may catalyze the formation of peroxymonocarbonate.<sup>4</sup>

Currently, two other hypotheses are being examined. The first hypothesis is examining the addition of hydrogen peroxide to a completed reaction. Stadtman et al.

detected a decrease in rate when adding more hydrogen peroxide to a completed reaction. They suggested that the formation of an unreactive metal species was the cause of the decrease in rate.<sup>19</sup> However, data collected for this study suggest the loss of bicarbonate seems to be one reason for the decrease. In one day, the concentration of bicarbonate decreased by half. When the reactions were performed with a borate buffer, the decrease is very slow but continuous, as seen in Figure 2-7. Another possible reason for the decrease in rate is the presence of phosphates in the hydrogen peroxide. Phosphates are used to stabilize hydrogen peroxide at high concentrations. Examination of the second reason for the decrease in rate of reaction is underway with distilled hydrogen peroxide.

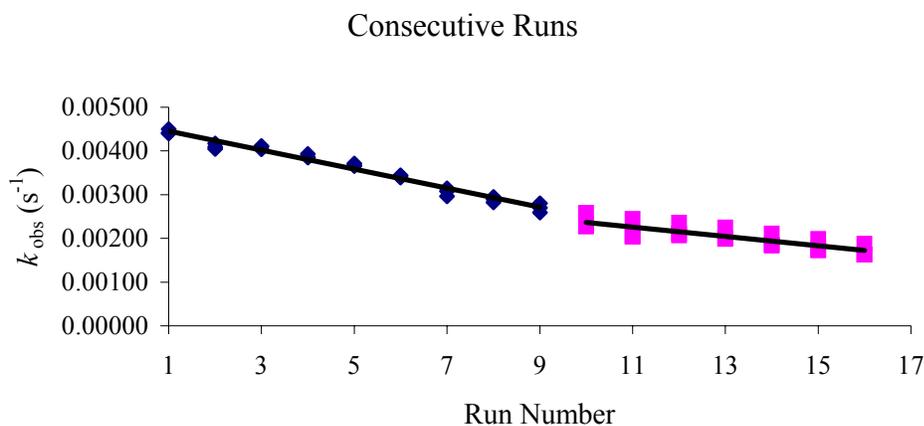


Figure 2-7: Continuous runs by the addition of hydrogen peroxide. A three-milliliter solution comprised 0.45 M sodium bicarbonate, 4  $\mu\text{M}$  manganese(II) sulfate, and 0.5 M borate buffer. The reactions were started by adding 25  $\mu\text{L}$  of 30% hydrogen peroxide and monitored using the UV-Vis. For every consecutive run, 20  $\mu\text{L}$  of 50% hydrogen peroxide was added to a completed reaction. The difference between the two lines is they were performed on different days.

The other hypothesis, proposed by the Richardson's group, focuses on the question concerning the second order dependence of bicarbonate. The formation of peroxymonocarbonate may consume one of the bicarbonate molecules, and the other bicarbonate molecule may bind the metal. If the second bicarbonate binds the metal, it

may be possible to change the rate order of bicarbonate by adding other metal chelators. If the rate order varies, the result would support the hypothesis. Currently, both different macrocycles and molecules that could bind in a manner similar to bicarbonate are being used to test the hypothesis. Initial results have not supported the hypothesis but more studies are being performed.

The study presented here demonstrated that some buffers, such as phosphate and HEPES, change the rate. One also must also be aware of all the contaminants within bicarbonate and hydrogen peroxide. Many salts contain both phosphates and carbonate and both anions are very insoluble with manganese(II).

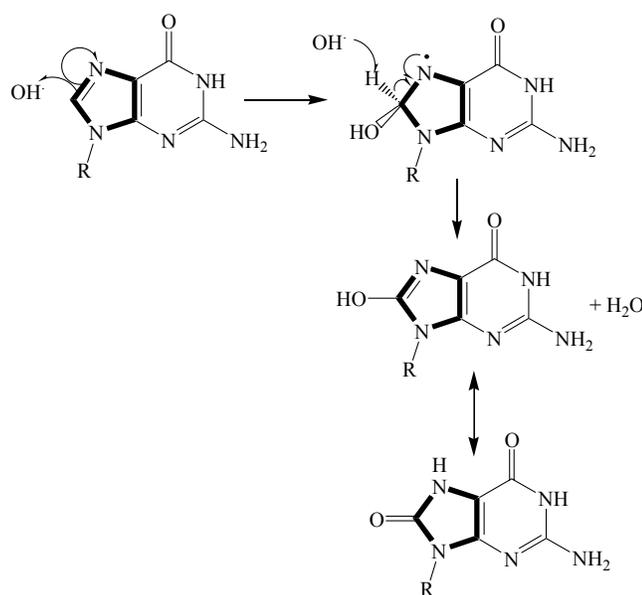
One buffer, boric acid/borate, has not been shown to change the rate. Using this buffer, the disproportionation of hydrogen peroxide has been analyzed. Both the rate order of manganese(II) and bicarbonate have been determined, and the values match reported values. Studies are continuing to propose a mechanism for the decomposition of hydrogen peroxide with manganese(II) and bicarbonate.

**Oxidation of DNA.** Using  $^1\text{H-NMR}$ , Bennett detected a reaction between hydrogen peroxide and linoleic acid in the presence of bicarbonate, forming an epoxide at each of its double bonds.<sup>16</sup> The result was an early observation of peroxymonocarbonate oxidization of biological molecules. From the result, Regino decided to study the oxidation of another biological molecule, guanosine monophosphate, with peroxymonocarbonate.<sup>10</sup> During most oxidations of guanosine monophosphate, 8-oxoguanosine forms [Scheme 2-2]. Another reason for choosing guanosine was its reduction potential, the lowest of all DNA bases, Table 1.<sup>23</sup>

Table 1: One-electron reduction potentials for nucleobases (Data as reported by Steenken and Jovanovic).<sup>23</sup>

Species <sup>a</sup>	$E^\circ$ (vs. NHE), V	PH
rG	1.29	7
rA	1.42	7
dC	1.6	7
dT	1.7	7
r(8-oxoG)	0.58	8
r(8-oxoA)	0.92	8
r(5-OH-C)	0.62	8
r(5-OH-U)	0.64	8
Tryptophan	1.01	8
RS·	0.75	8

<sup>a</sup>In the list, r denotes ribonucleoside and d denotes deoxyribonucleoside moiety.



Regino's initial solution contained 1.0 M guanosine monophosphate, 1.0 M hydrogen peroxide and 1.0 M ammonium bicarbonate in a 0.5 M ammonium phosphate buffer at a pH of 8.3.<sup>10</sup> Using a High Pressure Liquid Chromatograph (HPLC) equipped with electrospray injection (ESI), one product detected had a molecular weight 16 atomic mass units higher than guanosine monophosphate. The product may be 8-oxoguanosine monophosphate. Regino determined that manganese(II) is required for the reactions. She

also developed a HPLC method to separate guanosine monophosphate from the other oxidation products.

Regino's work was reproduced in order to study the oxidation of DNA. The addition of 5  $\mu\text{M}$  manganese(II) to the reaction conditions was included due to the requirement of manganese(II) for the reaction. After starting a reaction, aliquots of the reaction were diluted. The samples were then analyzed using her HPLC method. The results did not match the previously obtained chromatogram, possibly due to the metal addition. The additional metal may have affected the reaction. Therefore, a reaction without the added metal was performed. The resulting chromatogram still did not match the chromatogram reported by Regino (Figure 2-8).

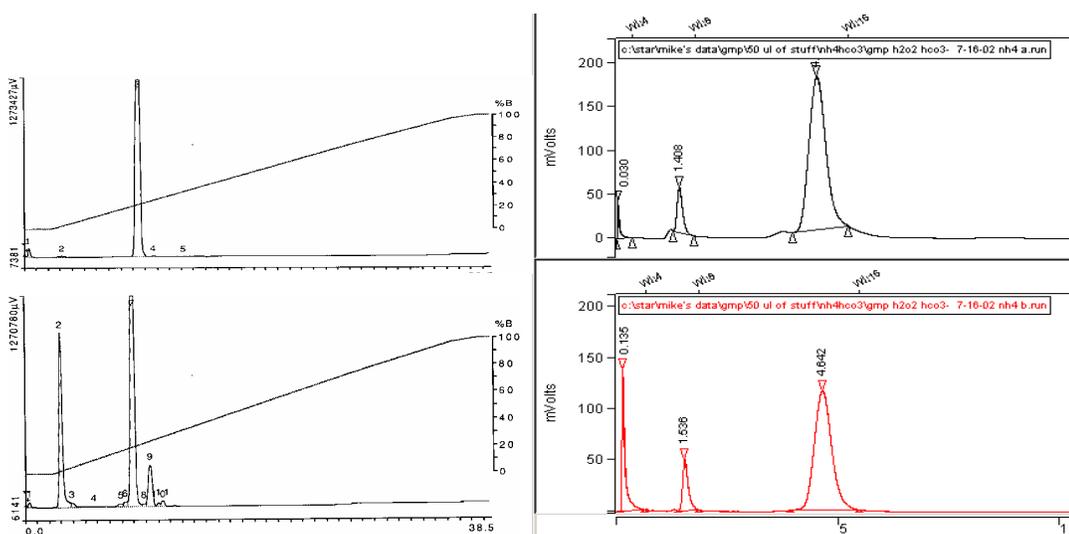


Figure 2-8: Comparison of the obtained chromatograms: Regino and the ones obtained for the current study. The two chromatograms on the left were obtained by Regino.<sup>10</sup> The two chromatograms on the right obtained for the study presented here. The top chromatograms were at before a reaction. The bottom chromatograms were after an hour. Both reactions comprised 1.0 M guanosine monophosphate, 1.0 M hydrogen peroxide and 1.0 M ammonium bicarbonate in a 0.5 M ammonium phosphate buffer at a pH of 8.3.

While working out the problems with the HPLC, other questions had to be answered. One of which was 'Which of the absorbencies detected was possibly due to 8-

oxoguanosine monophosphate eluting the column?’ To answer the question, I endeavored to produce 8-oxoguanosine monophosphate with an iron complex, but was unsuccessful.<sup>34</sup> I then attempted to purchase 8-oxoguanosine monophosphate. Unfortunately, no commercial source was available.

However, 8-oxoguanosine, a derivative of 8-oxoguanosine monophosphate, was commercially available. The only structural difference is that the phosphate group off the sugar is exchanged for an alcohol group (Figure 2-9). The structural change effects the solubility in water. Since 8-oxoguanosine monophosphate (a oxidized product of guanosine monophosphate) is negatively charged at the pH that the reaction is performed, and 8-oxoguanosine (a oxidized product of guanosine) is not, 8-oxoguanosine is less soluble. The lower concentration of 8-oxoguanosine will decrease the rate of reaction, but the oxidation should proceed in the same manner since no detected reaction involves the sugar ring.

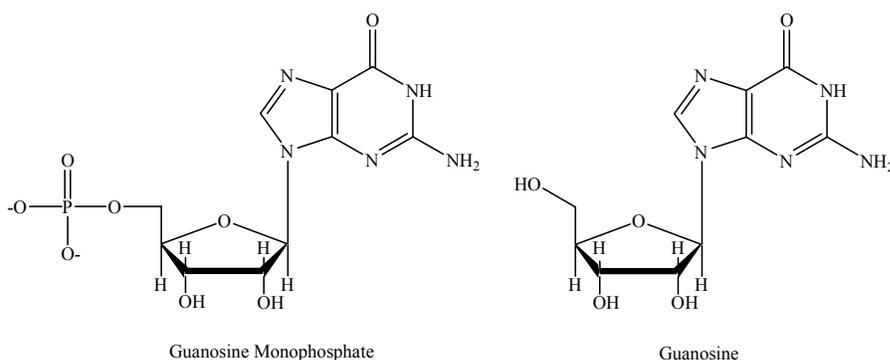


Figure 2-9: Structures of guanosine monophosphate and guanosine

Many published methods describe a way to separate guanosine and 8-oxoguanosine using the HPLC. Guanosine and its oxidized product were separated by using the method published by Kino. The resulting chromatogram is seen in Figure 2-10.<sup>24</sup> The elution times for guanosine and 8-oxoguanosine are 23 minutes and 25 minutes, respectively.

With separation and the retention times known, a reaction was performed and analyzed with the method.

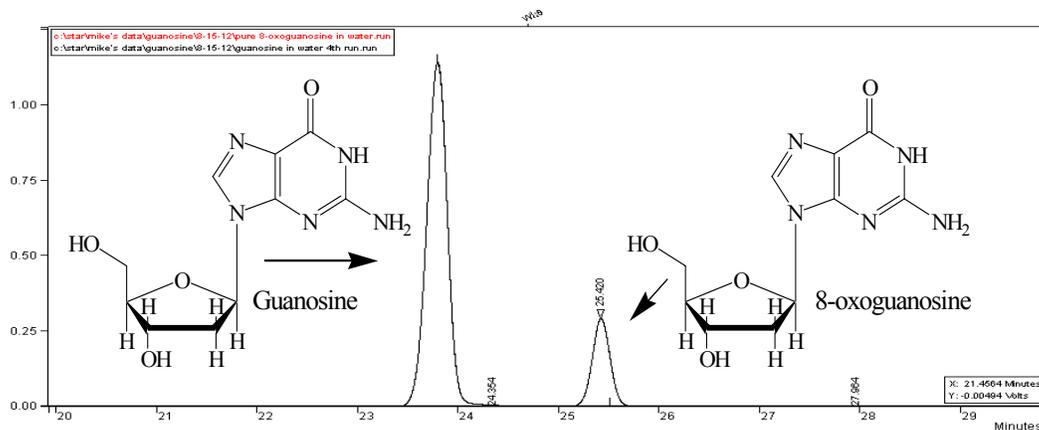


Figure 2-10: Using a published method, the retention times for guanosine and 8-oxoguanosine are 23 and 25 min respectively.<sup>24</sup>

After a reaction of 1mM guanosine, 1mM hydrogen peroxide, 1mM ammonium bicarbonate, and 5  $\mu$ M manganese(II), buffered in 0.5 M ammonium phosphate reacted for 30 minutes, the reaction was quenched. From a diluted aliquot of the reaction, 25  $\mu$ L were injected onto the HPLC. We detected a molecule eluting the column with the same retention time as that of pure guanosine, as well as other oxidized products, but no molecule eluting at the same retention time as that of pure 8-oxoguanosine. Therefore, using the same reaction conditions, a second reaction was started and quenched after 10 minutes, 20 minutes sooner than in the previous reaction, in the hopes of detecting 8-oxoguanosine. A second sample was injected into the HPLC. The resulting chromatogram showed fewer products being formed, but still no product with the same retention time as pure 8-oxoguanosine.

Detection of 8-oxoguanosine was not possible under the initial conditions. In an attempt to detect the desired product, the concentrations of the reactants were varied. The ratio of hydrogen peroxide to guanosine was varied from 1:1 up to 20:1, and samples of

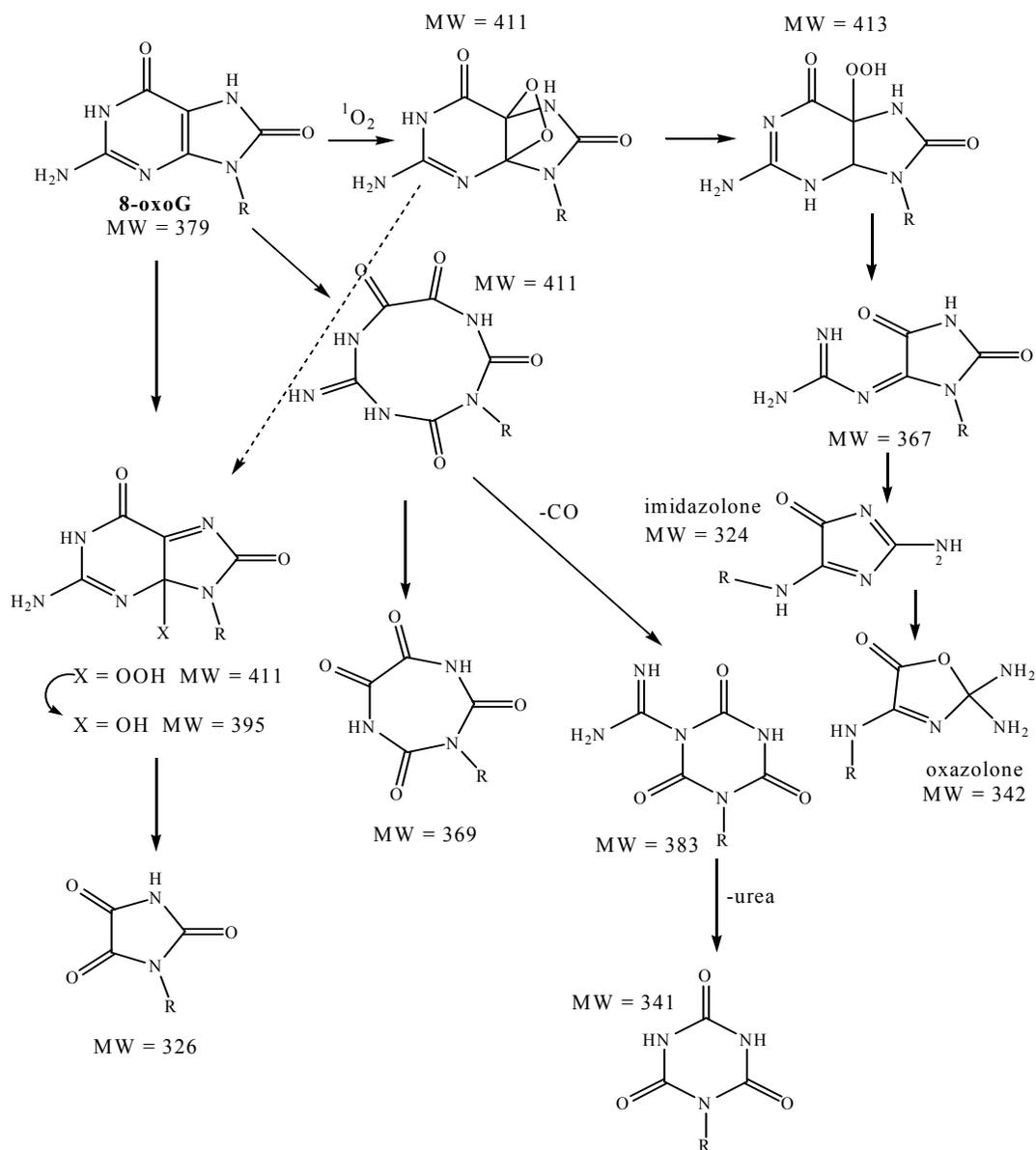
these reactions were injected into the HPLC. During the oxidation reactions, 8-oxoguanosine was never detected. Compared to guanosine, 8-oxoguanosine has a lower reduction potential, possibly resulting in a faster oxidation of 8-oxoguanosine. The faster oxidation would limit the concentration in solution, making it harder to detect the product. Besides the reduction potential of 8-oxoguanosine, the oxidation of 8-oxoguanosine can occur at more than one position, increasing its oxidation rate. Shown in Scheme 2-3 are the proposed pathways for 8-oxoguanosine to be oxidized by hydroxyl radicals.<sup>25</sup>

Currently, other reported methods of detection are not available to us. Kinetically, the oxidation reaction was difficult to study. A different substrate was sought. Hoping to continue studying the oxidation of guanosine, a molecule proposed to oxidize in a similar manner was obtained. The new substrate was an amino acid. The oxidized product is detected near the active sites of some enzymes.<sup>26</sup>

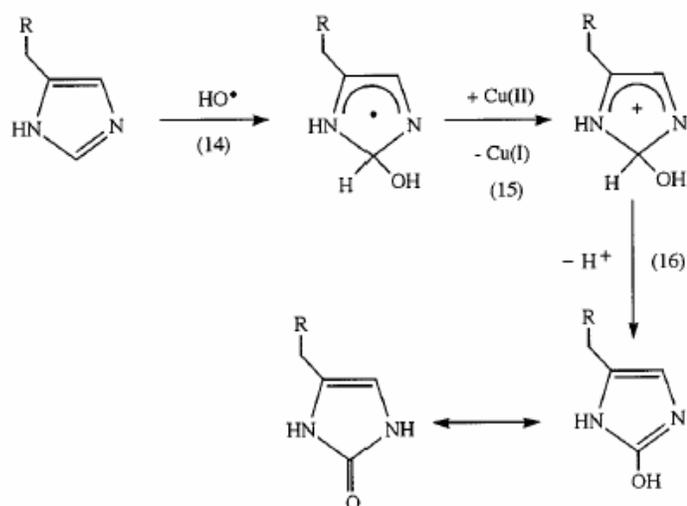
**Oxidation of amino acids.** Of the twenty amino acids, histidine was the substrate of interest. A proposed mechanism for the oxidation of the imidazole ring on histidine by the hydroxyl radical includes a pathway similar to the pathway to form 8-oxoguanosine from guanosine [Scheme 2-4].<sup>26</sup> If oxidation of the imidazole ring could be followed, the pathway by which the oxidation of the DNA base occurs may be better understood.

A histidine standard was obtained using Proton Nuclear Magnetic Resonance (<sup>1</sup>H-NMR). Employing the oxidation method for guanosine, oxidation of histidine was completed. The reaction was also analyzed using <sup>1</sup>H-NMR. As seen in Figure 2-11, the NMR spectrum after a reaction shows more peaks than does the standard of spectrum of histidine. The peak at 4.60 is from deuterate of water (HOD). The peaks found downfield

from the HOD peak are protons from both the side-chain and the products of the oxidation of the side-chain. The peaks found upfield from the HOD peak are from the protons from the peptide of histidine and from the oxidized products of both the backbone and side-chain of histidine. This result makes it difficult to determine the



Scheme 2-3: The pathways of the oxidation of guanosine (R = sugar backbone).<sup>25</sup>



Scheme 2-4: Oxidation of the imidazole ring on histidine.<sup>26</sup>

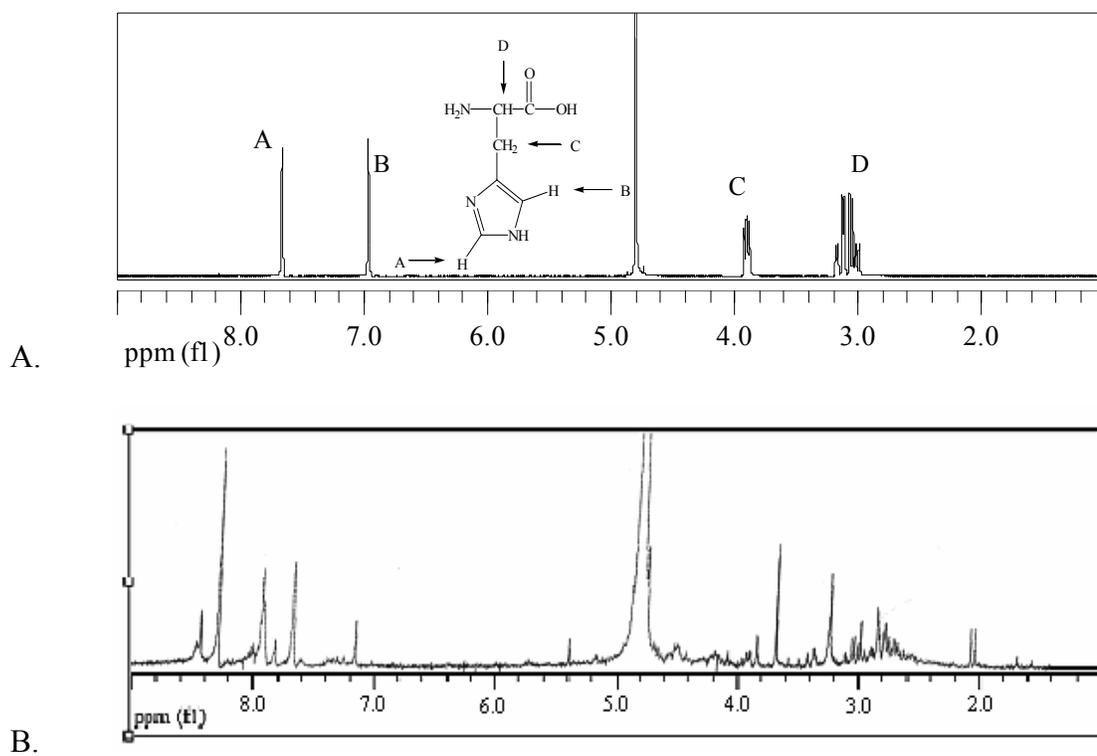


Figure 2-11: Oxidation of histidine. (A) A NMR standard of 0.1 M histidine, buffered in 0.5 M phosphate buffer near pH of 8.3. (B) Oxidation of histidine. The reaction consisted of 0.1 M histidine 2.0 M hydrogen peroxide, 1.0 M bicarbonate, 5 mM Manganese, buffered in 0.5 M phosphate buffer near pH of 8.3.

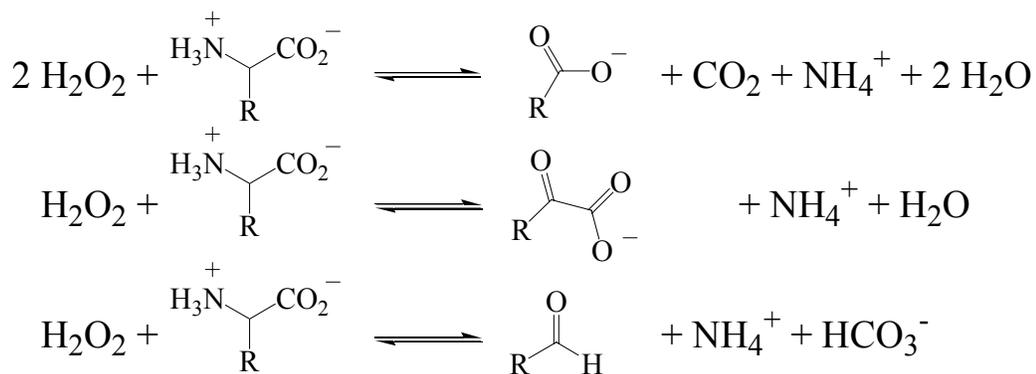
products using the NMR. Some published studies listed possible products from the oxidation of the imidazole on histidine. The listed products included 2-oxohistidine, aspartic acid, and asparagine.<sup>7</sup> For the oxidation of the side-chain, the pathway to form the products is proposed to proceed through two consecutive reactions. The first is a deamination reaction, and the second is a decarboxylation reaction.<sup>35</sup>

The first set of experiments examined the oxidation of the backbone using an amino acid with a less reactive side-chain. Glycine has the simplest side-chain of all the amino acids, but peaks would not appear in the <sup>1</sup>H-NMR for some of the proposed oxidation products.<sup>27</sup> Therefore, it was not chosen to be studied. The next simplest amino acid is alanine. The oxidization of alanine can be analyzed by <sup>1</sup>H-NMR. Therefore, the pathway by which the backbone of an amino acid oxidizes will be examined with alanine. A standard of alanine was then obtained using <sup>1</sup>H-NMR.

First, a solution of alanine and manganese(II) was analyzed with the NMR to detect the presence of broadened peaks due to the addition of metal to the solution; the NMR spectrum did not show any broadened peaks.<sup>28</sup> Then, two background reactions were performed. The first examined if a reaction occurred between bicarbonate and alanine. The second tested for a reaction between hydrogen peroxide and alanine. In both cases, no reaction was detected, as shown in Figure 2-12. After analyzing the background reactions, alanine was reacted with hydrogen peroxide and bicarbonate. A reaction was detected. Two different hypotheses were formed from this result. One hypothesis requires no metal for the oxidation of alanine by peroxydicarbonate. The other hypothesis is the requirement of metal for the oxidation reaction to proceed, which would be similar to the oxidation of guanosine monophosphate. Even though metal was not added,

manganese is one contaminant found in sodium bicarbonate. The metal contaminant may have been the cause of the detected reaction. To further examine the second hypothesis, 5  $\mu\text{M}$  metal was added to the reaction. As shown in Figure 2-12, a higher concentration of products was detected. This result supports the second hypothesis, requiring metal for the oxidation reactions. More experiments are needed to examine the importance of metal during these oxidation reactions.

The first step to determine the products was examining Stadtman's papers.<sup>2;20;35</sup> In his papers, he proposed reactions for the detected products.<sup>2</sup> These reactions are shown in Scheme 2-5. One product he reported was an aldehyde. In the case of alanine oxidation, ethanal is the aldehyde that forms. If ethanal forms, a peak should appear between 9 and 10 in the  $^1\text{H-NMR}$  spectrum. When analyzing the reactions, no peaks were detected downfield from the HOD peak. The boiling point of ethanal is below room temperature, and the reactions were performed open to the air. Both of these suggest the evaporation of ethanal may occur prior to analysis. Stadtman and coworkers performed their reaction in a closed container, eliminating the possibility for ethanal to evaporate.



Scheme 2-5: Reactions that Stadtman proposed to obtain the products they detected. If alanine was the amino acid being oxidized, R = Methyl.<sup>2</sup>

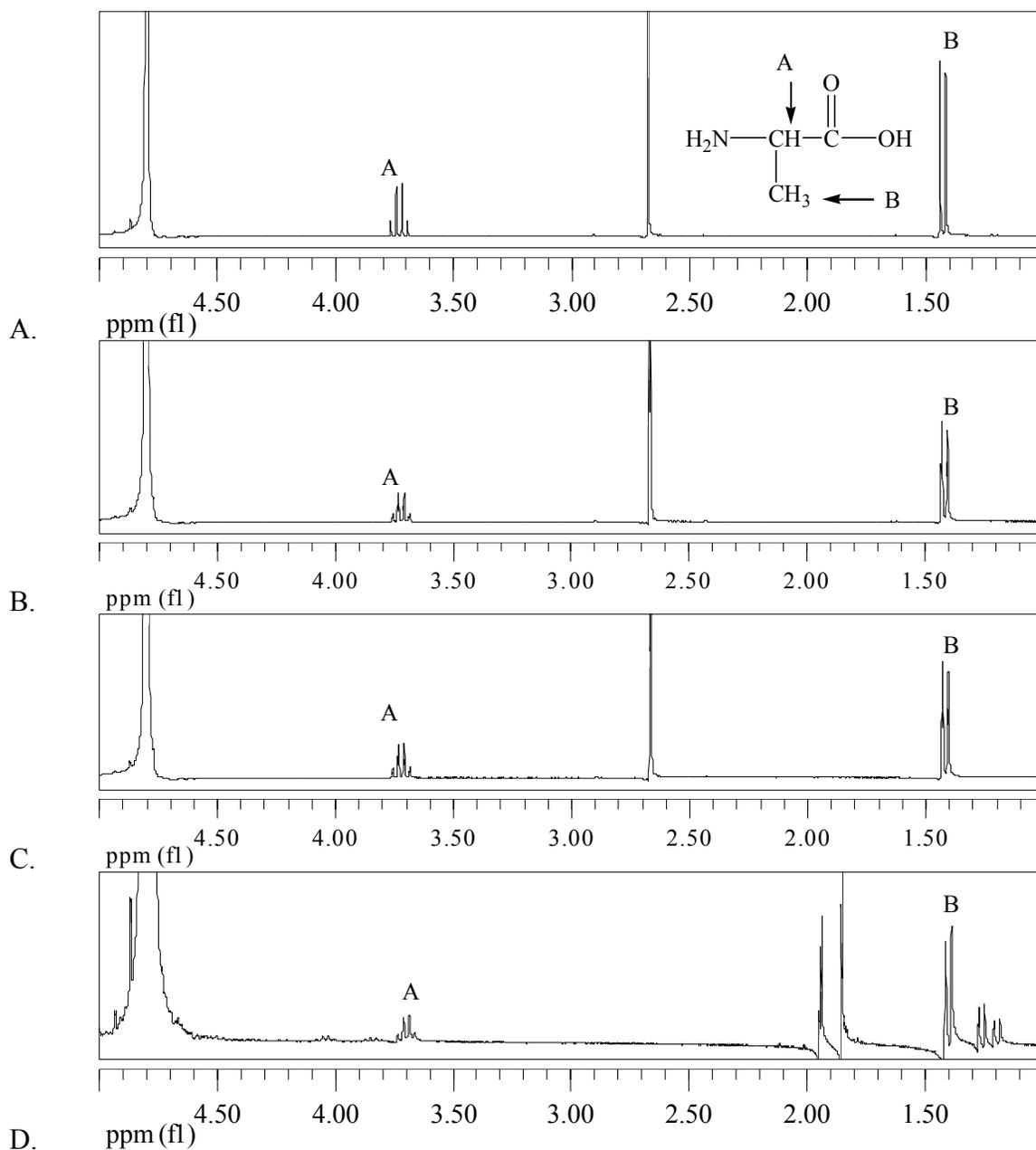


Figure 2-12: Oxidation of Alanine. (A) 0.10 M Alanine, (B) 0.10 M Alanine with 1.0 M hydrogen peroxide after 24 hours, (C) shows 0.10 M alanine with 0.5 M bicarbonate, and (D) shows 0.10 M alanine with a mixture of 5  $\mu\text{M}$  manganese, 0.5 M ammonium bicarbonate, and 1.0 M hydrogen peroxide. All of the mixtures were buffered in phosphate at 8.3 and the solvent is  $\text{D}_2\text{O}$ .

To determine if an aldehyde forms, a different amino acid was studied. To produce an aldehyde with a higher boiling point, an amino acid with a longer side-chain than that of alanine was examined. When oxidizing leucine, the resulting aldehyde formed (isovaleraldehyde) has a boiling point of 90°C.

Another concern was eliminated at the same time. This other worry dealt with over-oxidation of the amino acids. During the oxidation of both histidine and alanine, the concentration of the hydrogen peroxide was greater than ten times the concentration of the amino acid. A Fenton type reaction may be possible with the excess hydrogen peroxide. To eliminate this concern, experiments using Stadtman's lab procedure were performed.<sup>2</sup>

However, with the new procedure came a new concern. They used a carbon dioxide-rich atmosphere above the solution to buffer the oxidations.<sup>2</sup> During the decomposition studies, Statman's lab detected a different rate order for bicarbonate with the addition of carbon dioxide to the atmosphere above the solution. Therefore, a reaction with the addition of carbon dioxide to the atmosphere above the solution may change the reactivity.

A series of experiments were devised to test the effects, if any, of a carbon dioxide-rich atmosphere. First, a series of experiments were performed with carbon dioxide added to the atmosphere. The first experiment completed reproduced experiments lead by Stadtman.<sup>2</sup> After two hours, the solution was analyzed by two different methods. The first method used the HPLC. Stadtman and coworkers had reported using the HPLC as a means to determine what products formed. When performing the reported HPLC method, none of the molecules retention times were similar when comparing runs.

The other method used was the NMR. Besides leucine, the three reported products were purchased, and  $^1\text{H-NMR}$  standards for all of these molecules were obtained.<sup>2</sup> Two hours after starting a reaction, it was analyzed with  $^1\text{H-NMR}$ . The resulting spectrum is shown in Figure 2-13. Some of the new peaks have the same chemical shift as the protons from two of the products detected by Stadtman and coworkers (4-methyl-2-oxovaleric acid and isovaleric acid). When increasing the concentration of carbon dioxide, an unexpected result was obtained. The change in the concentration of carbon dioxide caused the concentration of isovaleric acid to increase, and the concentration 4-methyl-2-oxovaleric acid to decrease.

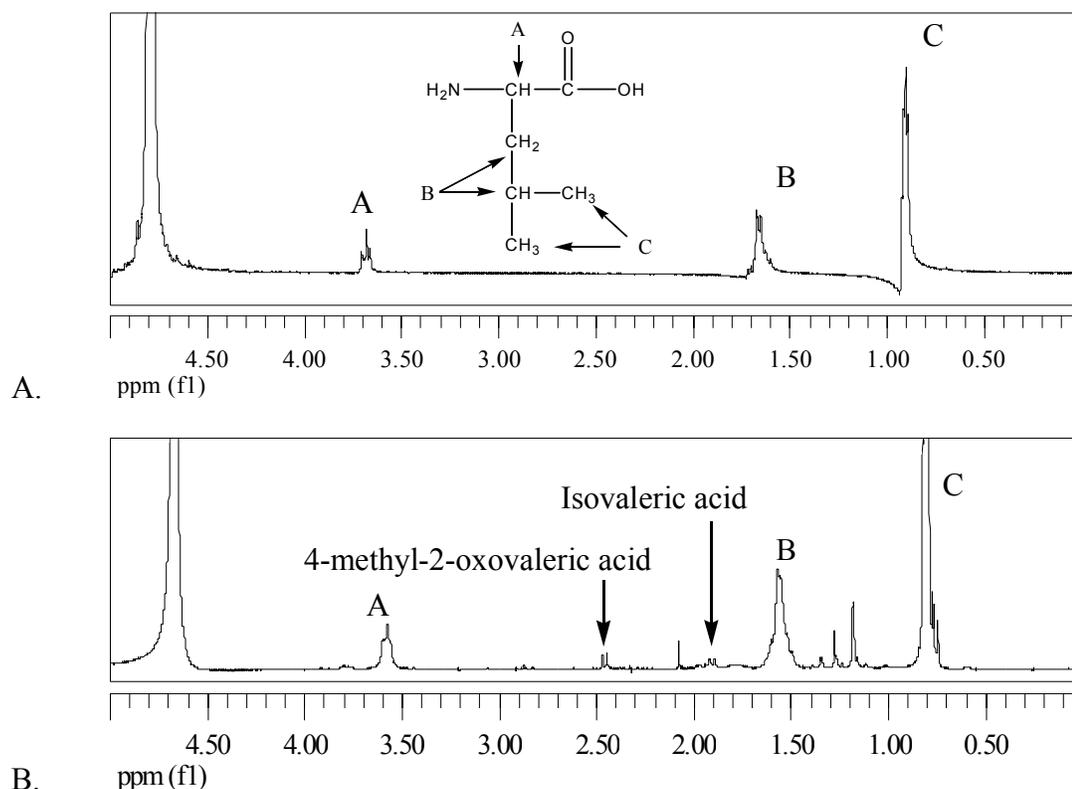
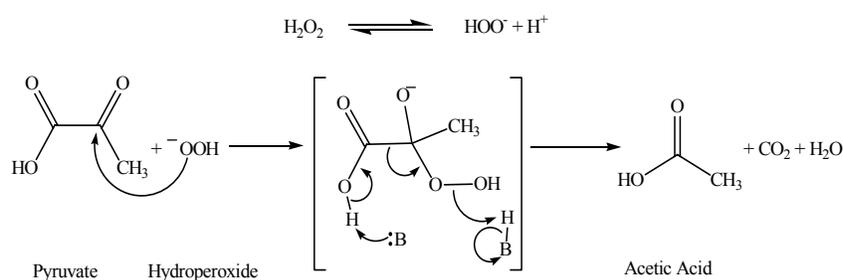


Figure 2-13: Oxidation of leucine (buffered in carbon dioxide and bicarbonate). (A) Shows leucine in  $\text{D}_2\text{O}$ , (B) shows the oxidation of leucine under an atmosphere of 15%  $\text{CO}_2$  and 85%  $\text{N}_2$ . The concentrations for both reactions were 50 mM leucine, 30 mM hydrogen peroxide, 23.5 mM sodium bicarbonate, and 50  $\mu\text{M}$  manganese(II).<sup>2</sup>

Through pH changes, the varying concentrations of the two products can be explained. The solution becomes more acidic as the concentration of carbon dioxide above the solution increases. The result of increasing acidity makes hydroperoxide ( $\text{HOO}^-$ ) less abundant in the solution. Hydroperoxide is hypothesized to decarboxylate  $\alpha$ -keto acids, resulting in the formation of a carboxylic acid [Scheme 2-6].<sup>29-33</sup> In the reaction, 4-methyl-2-oxovaleric acid is the  $\alpha$ -keto acid, and isovaleric acid is the resulting carboxylic acid.



Scheme 2-6: The proposed decarboxylation reaction.<sup>30</sup>

To demonstrate the decarboxylation reaction, 4-methyl-2-oxovaleric acid was mixed with varying amounts of hydrogen peroxide. As the ratio of hydrogen peroxide to 4-methyl-2-oxovaleric acid increased, more isovaleric acid formed. This result is shown in Figure 2-14. Another technique of analyzing the decarboxylation reaction is with Carbon-13 Nuclear Magnetic Resonance ( $^{13}\text{C}$ -NMR). Two  $^{13}\text{C}$ -NMRs were taken. The first was of a saturated solution of bicarbonate, and the second was after the decarboxylation reaction completed. The chemical shift of the carbon in bicarbonate appears just above 160. As shown in Figure 2-14, after analyzing the reaction, a peak that appeared just above 160 is not present in the standard of isovaleric acid. The peak at 160 appears because of the reaction between carbon dioxide and water [Scheme 2-7].



Scheme 2-7: Hydration of  $\text{CO}_2$ , forming bicarbonate.

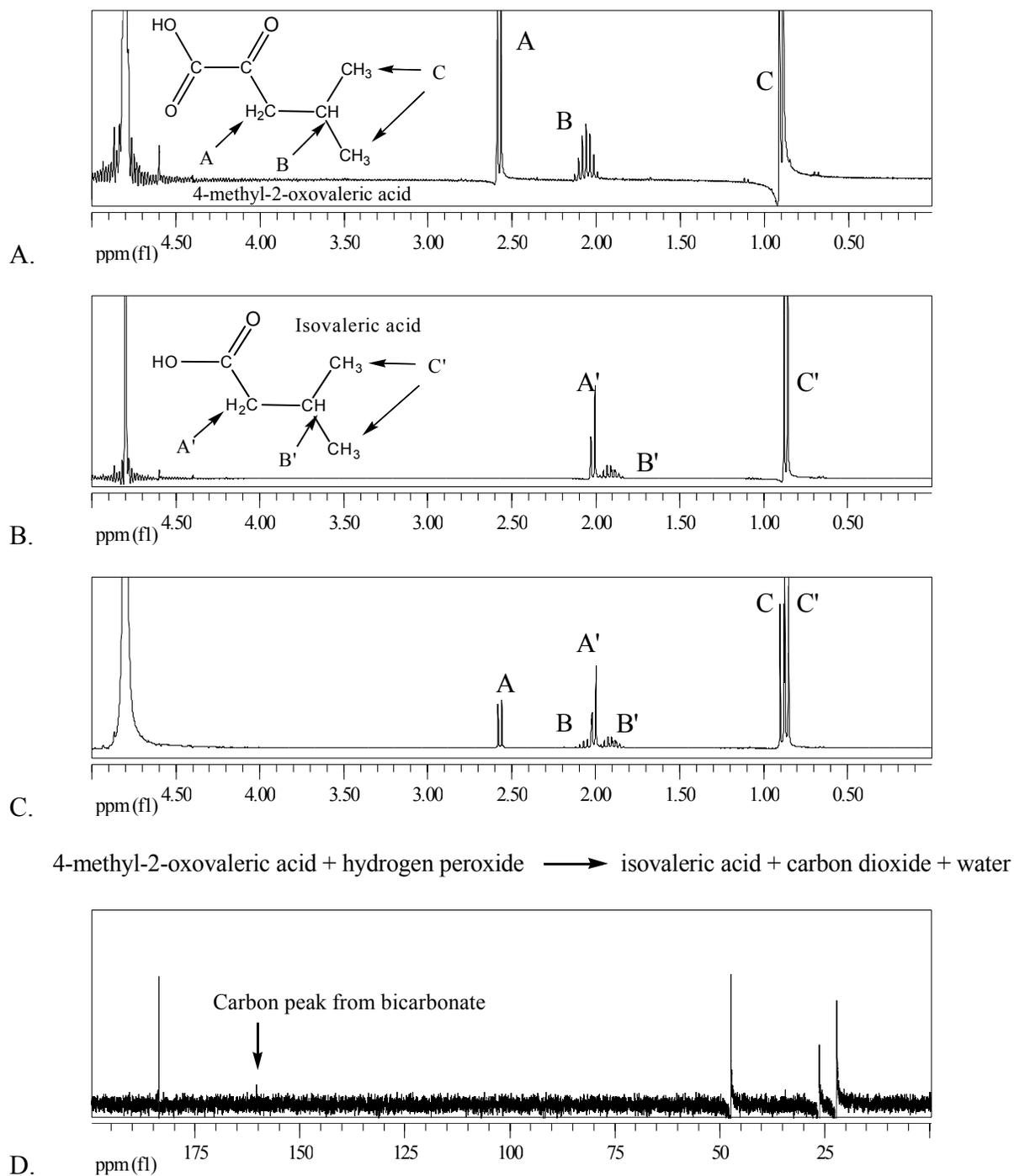


Figure 2-14: Decarboxylation reaction. (A) 50 mM 4-methyl-2-oxovaleric acid, (B) 50 mM isovaleric acid, (C) and (D) shows the decarboxylation of 4-methyl-2-oxovaleric acid ( $^1\text{H}$  and  $^{13}\text{C}$ ) using 50mM 4-methyl-2-oxovaleric acid, and either 30 mM hydrogen peroxide (C) or 60 mM hydrogen peroxide (D) in a phosphate buffer, pH 8.3. All reactions preformed in  $\text{D}_2\text{O}$ .

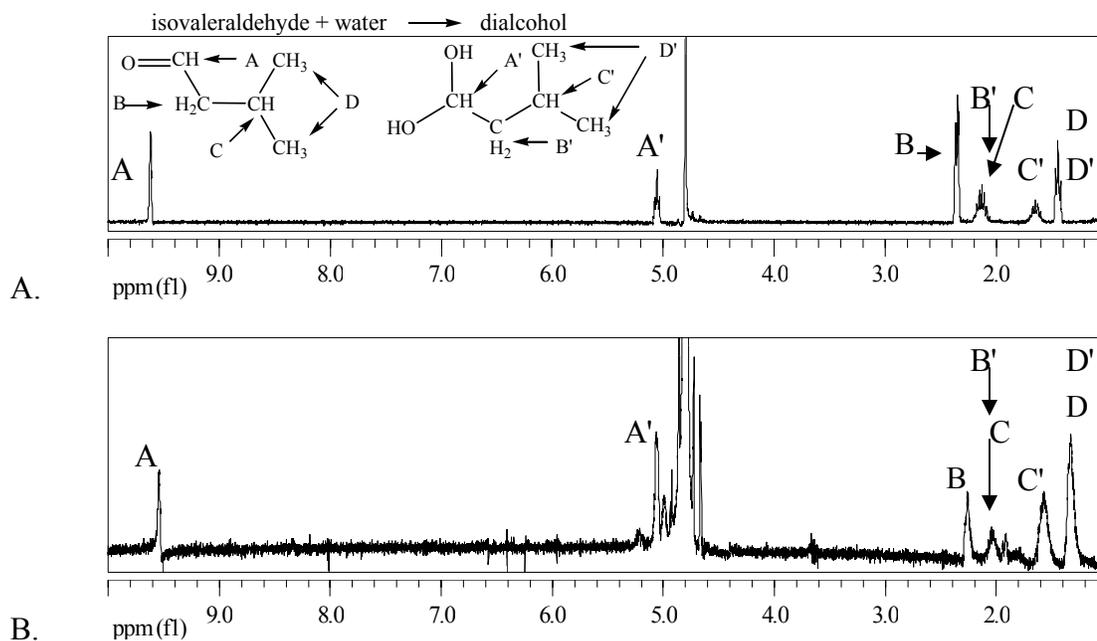


Figure 2-15: Reaction with isovaleraldehyde. (A) NMR standard of isovaleraldehyde. The concentration of the aldehyde is .5 M. The buffer used comprised borate, with the pH was near 8.34. (B) Oxidation of isovaleraldehyde, where the concentrations were 50 mM aldehyde, 30 mM hydrogen peroxide, 23.5 mM sodium bicarbonate, and 50  $\mu$ M manganese(II). This reaction was buffered in phosphate buffer, with the pH near 8.3.

To determine if any other reactions occur, 4-methyl-2-oxovaleric acid and isovaleric acid were reacted separately under the same conditions used to oxidize leucine. Aside from the decarboxylation reaction, no other reaction was detected.

A standard of isovaleraldehyde was also analyzed. As shown in Figure 2-15, peaks that appear in the NMR spectra after an oxidation of leucine have the same chemical shifts as protons showed in the standard of aldehyde, supporting Stadtman's group results.<sup>2</sup> However, the peak between 9 and 10 found in the NMR spectrum of the aldehyde is not detected after a reaction. Detection of the peak may be difficult due to a reaction between isovaleraldehyde and water, forming a dialcohol. Isovaleraldehyde was also oxidized similar to leucine. The only product detected has the same chemical shifts as peaks appear in the NMR spectrum of a standard of isovaleric acid.

After analyzing the carbon dioxide and bicarbonate buffered oxidation reactions, a reaction buffered with phosphate was performed. After two hours, a single peak appeared. Originally, the reaction was thought to be completely finished. However, recently obtained results show the rate of the decomposition of hydrogen peroxide decreases with addition of phosphates. The theory presented was that the reaction did not run to completion in the two hours allotted. In a subsequent trial, the reaction was allowed to proceed for 24 hours. The reaction was then analyzed by  $^1\text{H-NMR}$  and the resulting spectrum is shown in Figure 2-16. Some similar results were obtained between the different reactions. However, there are some differences. There is a new peak above 3.5 and the chemical shifts of the protons between 1.0 and 1.5 are spaced differently. More experiments are needed to determine why different reactions occur with the different buffers.

Three hypotheses are supported from studying the oxidation of biological molecules. The first is that phosphates effect the rate of reaction. The second is that the decomposition reaction can be used to study the oxidation reactions. The third is everything in the solution can effect the rate of these reactions.

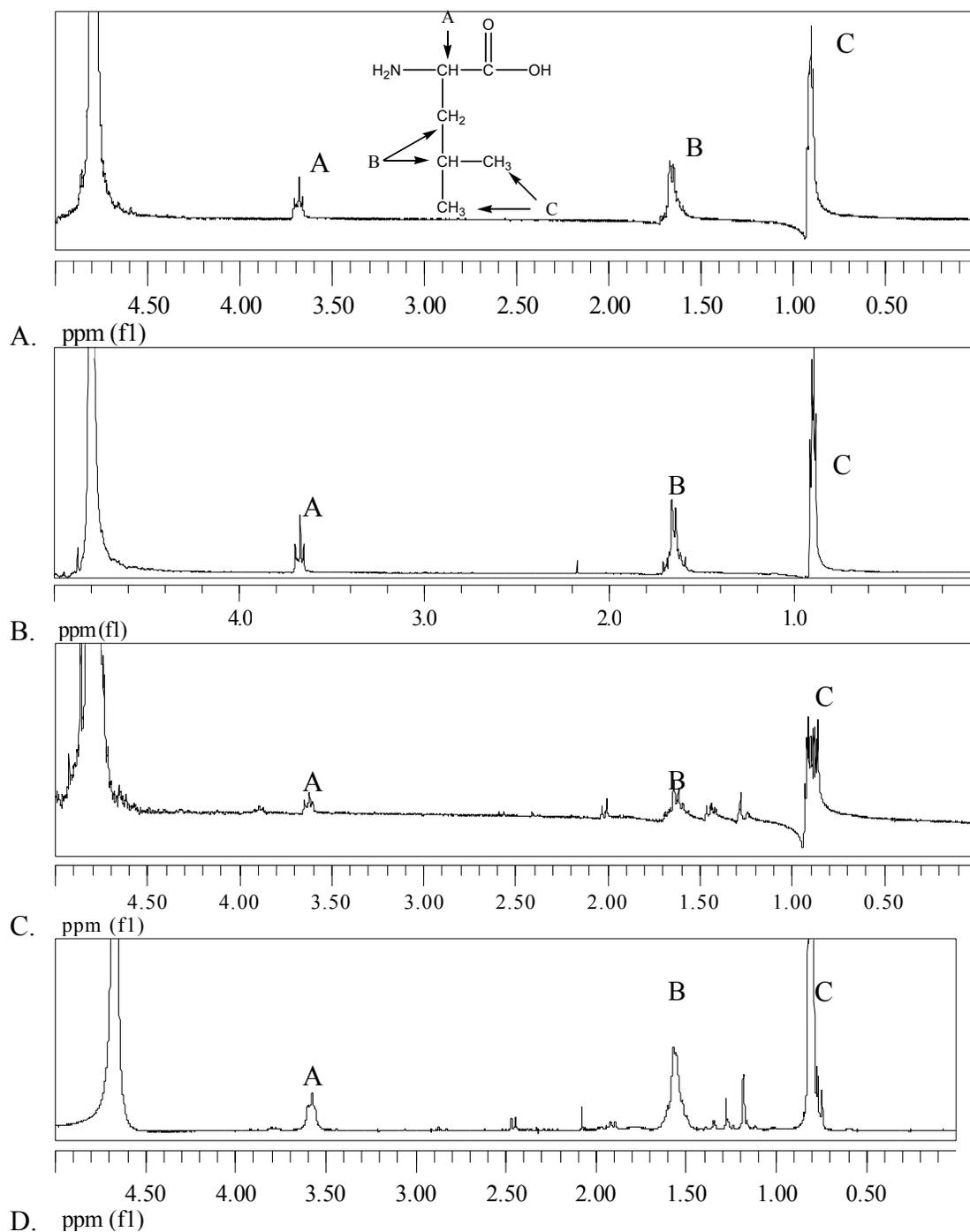


Figure 2-16: Comparing oxidation of leucine in two different buffers. A) Shows leucine in  $\text{D}_2\text{O}$ , (B) shows oxidation of leucine after two hours open to the air and buffered with sodium phosphate at pH 8.3, (C) shows oxidation of leucine after twenty four hours open to the air and buffered with sodium phosphate at pH 8.3, (D) shows the oxidation of leucine under an atmosphere of 15%  $\text{CO}_2$  and 85%  $\text{N}_2$ . The concentrations for both reactions were 50 mM leucine, 30 mM hydrogen peroxide, 23.5 mM sodium bicarbonate, and 50  $\mu\text{M}$  manganese(II).

## CHAPTER 3 CONCLUSION

The oxidation reactions by peroxydicarbonate have been examined through the study of the disproportionation of hydrogen peroxide. From decomposition studies, some buffers have been shown to affect the rate of reaction. Two of these buffers are phosphate and HEPES.

Examination of a borate buffer was also undertaken. From obtained results, no effect on the rate of the decomposition of hydrogen peroxide has been detected. Using borate to buffer the solutions near 8.3, the rate orders for manganese(II) and bicarbonate were determined. The results were similar to the published values, first order for manganese(II) and second order for bicarbonate.<sup>18</sup> In Richardson's lab, two hypotheses are currently being examined. The first hypothesis is studying the effects of adding hydrogen peroxide to a completed reaction. The second hypothesis involves the importance of the second order dependence in bicarbonate.

Also presented here was the oxidation of biological molecules. Oxidation of some biological molecules, such as guanosine monophosphate, by peroxydicarbonate requires manganese(II). HPLC was used to study the oxidation reaction, and the product of interest was 8-oxoguanosine monophosphate. The separation of the oxidation products was inconclusive, and a derivative, guanosine, was then investigated.

Separation and detection of guanosine and 8-oxoguanosine were obtained using the HPLC, monitoring at 254 nm.<sup>24</sup> While analyzing the initial reaction, 8-oxoguanosine was not detected. In an effort to detect the product, variations in the method of oxidation

were engaged. While varying reaction time and the amount of hydrogen peroxide, detection of 8-oxoguanosine was never obtained. The only obtained result was that different products appeared, possibly due to over-oxidation of guanosine and all of its oxidized derivatives.

Kinetically, guanosine oxidation became difficult to study due to the formation of many products. Oxidative reactions of another group of biological molecules, amino acids, commenced. One amino acid of interest was histidine. A published oxidation pathway proposed the oxidation of the imidazole ring on histidine similar to guanosine.<sup>6</sup> Histidine was oxidized in the presences of peroxymonocarbonate and metal. Upon further review of reported results, both the side chain and the backbone became oxidized.<sup>2</sup> To understand the pathway for the oxidation of the backbone, oxidation reactions of alanine and leucine were studied. A study of the oxidation reactions showed that both deamination and decarboxylation reactions occurred on the backbone of the amino acid.

Oxidation of amino acids buffered by phosphate or carbon dioxide and bicarbonate produces some similar products. However, there are detectable differences. One notable difference was that, after two hours, detection of more products occurred when carbon dioxide and bicarbonate buffered the reaction compared with the reactions buffered in phosphate. One possible explanation for the difference is that phosphates decrease the concentration of metal in solution, resulting in a decrease in rate of reaction.

Another difference is, after twenty-four hours, the detection of more products when buffering the reaction with phosphate. This supports a hypothesis. The addition of many molecules can effect the rate of reactions. To determine the additional products formed in the phosphate buffer reactions, more experiments will be examined. These results have

suggested that peroxymonocarbonate is not a molecule which could cause much oxidative stress.

## CHAPTER 4 CHEMICALS AND EQUIPMENT

Guanosine monophosphate, guanosine, L-leucine, DL-alanine, glycine, L-histidine, boric acid, HEPES, sodium HEPES, and EPPS were obtained from Aldrich (St. Louis) and used as received. Ammonium bicarbonate, sodium bicarbonate, sodium phosphate (monobasic, dibasic, and tribasic), and manganese(II) sulfate were obtained from Fisher (Fairlawn, NJ) and used without further purification. Hydrogen peroxide (both 30% and 50%) was obtained from Solvay Interlox Inc, and D<sub>2</sub>O was obtained from Cambridge Isotope Laboratories, Inc. The 8-oxoguanosine was obtained from Toronto Research Chemicals.

Two different High Pressure Liquid Chromatographs (HPLCs) were used during the experiments. One HPLC, bought from Rainin, was the HPXL model with a single wavelength detector. The other HPLC was a Prostar Varian HPLC with a photo-diode array detector. There were two different columns used during the experiments. The anion exchange column was a strong anion exchange column (Hydropore AX 83-603 4.6 x 1.5  $\mu\text{m}$ , 12  $\mu\text{m}$  300 Å) that was purchased through Rainin. The Supelco Discovery's 25cm x 4.6 mm C-18 reverse-phase column packed with 5  $\mu\text{M}$  endcapped, metal-free particles was also used during the experiments.

NMR spectra were obtained using a Gemini, a VXR, or a Mercury spectrometer operating at 300 MHz. The reactions were preformed in D<sub>2</sub>O, with spectra referenced to the residual solvent peak.

UV-Vis spectra were obtained using a Hewlett Packard UV-Vis spectrometer. The reactions were performed in H<sub>2</sub>O and the observed wavelength was 264 nm.

## CHAPTER 5 METHODS

**Phosphate dependence for the decomposition of hydrogen peroxide.** Before a reaction, 1.0 M sodium bicarbonate was put through a Chelex column and collected. In a quartz cuvette, a three-milliliter solution comprised 0.3 M sodium bicarbonate, 5  $\mu$ M manganese(II) sulfate, and 0 – 1.5 M phosphate buffer. To keep the ionic strength constant, 0 – 1.5 M sodium acetate was also added. The reactions were started by adding 25  $\mu$ L of 30% hydrogen peroxide and monitored using the UV-Vis.

**Preparing HEPES and EPPS buffers for the decomposition of hydrogen peroxide.** HEPES was used to buffer the reactions from 7.0 up to 7.9, and EPPS was used to buffer the reactions from 8.0 up to 8.6. Different amounts of sodium HEPES and HEPES were mixed to obtain the desired pH. A stock of the buffers was prepared to have a final concentration of 2.0 M. The concentration of the buffer in the reactions was diluted to 0.5 M.

**Oxidation of HEPES by peroxymonocarbonate.** A standard of HEPES was obtained by mixing 0.1 M HEPES with 0.5 M phosphate buffer, pH near 8.3. For the oxidation of HEPES, 0.1 M HEPES, 0.15 M hydrogen peroxide, .23 M bicarbonate, buffered in 0.5 M phosphate buffer near pH of 8.3.

**Preparing borate buffer for the decomposition of hydrogen peroxide.** To prepare a 1.0 M borate buffer, 6.184 grams of boric acid was added to 75 mL of water in a 100 mL flask. To this solution, 10 M sodium hydroxide was added until the pH of the solution was near 8.3. Water was then added again until the total solution was 100 mL.

**Borate dependence for the decomposition of hydrogen peroxide.** Before a reaction, 1.0 M sodium bicarbonate was put through a Chelex column and collected. In a three-milliliter solution, 0.4 M sodium bicarbonate, 3  $\mu$ M manganese(II) sulfate, and 0.2 – 0.5 M borate buffer were mixed. The reactions were started by adding 25  $\mu$ L of 30% hydrogen peroxide and monitored using the UV-Vis using a three-milliliter cell.

**Bicarbonate dependence for the decomposition of hydrogen peroxide.** Before a reaction, 1.0 M sodium bicarbonate was put through a Chelex column and collected. Mixed in a quartz cuvette, a three-milliliter solution comprised 0.05 – 0.8 M sodium bicarbonate, 5  $\mu$ M manganese(II) sulfate, and 0.5 M borate buffer. The reactions were started by adding 25  $\mu$ L of 30% hydrogen peroxide and monitored using the UV-Vis.

**Manganese(II) dependence for the decomposition of hydrogen peroxide.** Before a reaction, 1.0 M sodium bicarbonate was put through a Chelex column and collected. In a quartz cuvette, a three-milliliter solution that comprised 0.4 M sodium bicarbonate, 0 – 50  $\mu$ M manganese(II) sulfate, and 0.5 M borate buffer was mixed. The reactions were started by adding 25  $\mu$ L of 30% hydrogen peroxide and monitored using the UV-Vis.

**Ionic strength effect for the decomposition of hydrogen peroxide.** Before a reaction, 1.0 M sodium bicarbonate was put through a Chelex column and collected. In a quartz cuvette, a three-milliliter solution that comprised 0.4 M sodium bicarbonate, 4  $\mu$ M manganese(II) sulfate, and 0.5 M borate buffer was mixed. To increase the ionic strength, 0 – 1.5 M sodium acetate was added. The reactions were started by adding 25  $\mu$ L of 30% hydrogen peroxide and monitored using the UV-Vis.

**Many consecutive runs for the decomposition of hydrogen peroxide.** Before a reaction, 1.0 M sodium bicarbonate was put through a Chelex column and collected. In a quartz cuvette, a three-milliliter solution that comprised 0.45 M sodium bicarbonate, 4  $\mu$ M manganese(II) sulfate, and 0.5 M borate buffer was mixed. The reactions were started by adding 25  $\mu$ L of 30% hydrogen peroxide and monitored using the UV-Vis. For every consecutive run, 20  $\mu$ L of 50% hydrogen peroxide was added to a completed reaction.

**UV-Vis procedure to analyze the decomposition of hydrogen peroxide.** The reactions were analyzed at 264 nm (the shoulder from the hydrogen peroxide peak). At this wavelength, 0.1 M hydrogen peroxide gives an absorbance just above one. An absorbance of the reactions was taken every 16 seconds for most of the runs. For reactions containing more than 10  $\mu$ M manganese(II) sulfate, the absorbance was taken every second. For the reactions containing 0.2 M ammonium bicarbonate or less, the absorbance was taken every ten minutes.

**Oxidation of guanosine monophosphate.** First, 1.0 M hydrogen peroxide and 1.0 M ammonium bicarbonate were reacted together in 0.5 M ammonium phosphate (buffered at pH 8.3) for 20 minutes to allow the formation of peroxymonocarbonate. Then, 1.0 M guanosine monophosphate and 5  $\mu$ M manganese(II) sulfate were added to the reaction mixture. At room temperature, the reaction mixed for two hours. An aliquot of the reaction was diluted a million fold; 20  $\mu$ L of a diluted aliquot was injected into the HPLC. For the second reaction, the same procedure was used with the exception of the added metal to the reactions.<sup>10</sup>

**Detection of guanosine monophosphate on the HPLC using an anion exchange column.** Separation of guanosine monophosphate and the oxidized products were attempted using a strong anion exchange column. A binary pump system was used to introduce the mobile phase, which consisted of solvent A (5mM ammonium phosphate) and solvent B (750 mM ammonium phosphate) in a linear gradient, which went from 100% A to 100% B, over a 30 minute period. Both solvents were buffered at pH 4.0. The solvent was pumped at 1.0 mL/min and the reaction was monitored at 254 nm. Following the run, it took the column another 20 minutes to reequilibrate.<sup>10</sup>

**Standard of guanosine and 8-oxoguanosine.** Using the same HPLC method for analyzing the oxidation of guanosine reactions, 1 mM guanosine mixed with in 0.5 M ammonium phosphate buffer (pH 8.3). An aliquot of the reaction was diluted a thousand fold; 20  $\mu$ L of a diluted aliquot was injected into the HPLC. Using the same procedure for obtaining the retention time for guanosine, retention time for 8-oxoguanosine was found.<sup>24</sup>

**Oxidation of guanosine.** Similarly to the oxidation of guanosine monophosphate, 1.0 mM hydrogen peroxide and 1.0 mM ammonium bicarbonate were reacted together in 0.5 M ammonium phosphate buffer (pH 8.3) for 20 minutes. Then, 1.0 mM guanosine and 5  $\mu$ M manganese(II) sulfate were added to the reaction mixture. At room temperature, the reaction mixed for either 10 or 30 minutes. An aliquot of the reaction was diluted a thousand fold; 20  $\mu$ L of a diluted aliquot was injected into the HPLC. During the second set of reactions, hydrogen peroxide increased from 1 mM, up to 20 mM.

**Analyzing the oxidation of guanosine on the HPLC using a C18 reversed phase column.** A procedure for separating guanosine and 8-oxoguanosine was found in a paper published by Kino and Sugiyama. The procedure included a dual solvent system using 50 mM ammonium formate buffer and acetonitrile as solvents A and B, respectively. The HPLC procedure comprised 100% A from 0-5 min (isocratic), 100-93% A for 5-27 min (linear gradient), and 93% A for 27-35 min (isocratic). The flow rate was also one mL/min and the reactions were monitored at 254 nm. Each run took 45 minutes, followed by 20 minutes to allow the column to reequilibrate.<sup>24</sup>*Oxidation of histidine:* A histidine standard was prepared by mixing 0.1 M histidine with 0.5 M ammonium phosphate buffered (pH 8.3) in D<sub>2</sub>O. For the reactions, 2.0 M hydrogen peroxide and 0.5 M ammonium bicarbonate in 0.5 M ammonium phosphate (buffered at pH 8.3) were reacted together in D<sub>2</sub>O for 20 minutes. Then, 0.1 M histidine and 5 μM manganese(II) sulfate were added to the reaction mixture. At room temperature, the reaction mixed for twenty-four hours. Using <sup>1</sup>H-NMR, the reaction and the histidine standard were analyzed.<sup>10</sup>

**Oxidation of alanine.** An alanine standard was prepared by mixing 0.1 M alanine with 0.5 M ammonium phosphate buffered (pH 8.3) in D<sub>2</sub>O. 0.1 M alanine was mixed with 0.5 M ammonium phosphate buffered (pH 8.3) in D<sub>2</sub>O and either 0.5 M ammonium bicarbonate, 2.0 M hydrogen peroxide, or 5 μM manganese(II) sulfate. For the reactions, 2.0 M hydrogen peroxide and 0.5 M ammonium bicarbonate in 0.5 M ammonium phosphate (buffered at pH 8.3) were reacted together in D<sub>2</sub>O for 20 minutes. Then, 0.1 M alanine and 5 μM manganese(II) sulfate were added to the reaction mixture. All of the solutions were analyzed using <sup>1</sup>H-NMR after twenty-four hours

**Oxidation of leucine (phosphate buffered reactions).** A leucine standard was prepared by mixing 50 mM leucine with 0.5 M ammonium phosphate buffered (pH 8.3) in D<sub>2</sub>O. For the reactions, 30 mM hydrogen peroxide and 23.5 mM ammonium bicarbonate in 0.5 M ammonium phosphate (buffered at pH 8.3) were reacted together in D<sub>2</sub>O for 20 minutes. Then, 50 mM leucine and 50 μM manganese(II) sulfate were added to the reaction mixture. Open to the air, the reaction mixed for either two hours or twenty-four hours at room temperature. Using <sup>1</sup>H-NMR, the reaction and the leucine standard were analyzed.

**Oxidation of leucine (carbon dioxide / bicarbonate buffer).** Berlett et al. reported procedure was used for these reactions. Before a reaction, a tank was filled with both carbon dioxide and nitrogen. The percent of carbon dioxide varied from 10%, up to 20%. In a closed container, 30 mM hydrogen peroxide and 23.5 mM ammonium bicarbonate mixed in D<sub>2</sub>O for 10 minutes while bubbling the gas mixture through the solution. Then, 50 mM leucine and 50 μM manganese(II) sulfate were added to the reaction mixture. Bubbling continued until the gas tank was empty.<sup>2</sup> After two hours at room temperature, the reactions analyzed using <sup>1</sup>H-NMR.

**Decarboxylation of 4-methyl-2-oxovaleric acid.** The standard of 4-methyl-2-oxovaleric acid was obtained by mixing 50 mM 4-methyl-2-oxovaleric acid and 0.5 M ammonium phosphate buffered (pH 8.3) in D<sub>2</sub>O. The standard of isovaleric acid was obtained using the same method. The standard of sodium bicarbonate was obtained by taking a <sup>13</sup>C-NMR of a saturated solution of bicarbonate in D<sub>2</sub>O. In a NMR tube, 50 mM 4-methyl-2-oxovaleric acid and either 30mM or 60 mM hydrogen peroxide were mixed and buffered in sodium phosphate at pH 8.3.

**NMR Work.** All of the  $^1\text{H}$ -NMR spectra were taken in  $\text{D}_2\text{O}$  and referenced to the residual solvent peak. The  $^{13}\text{C}$ -NMR of the decarboxylation was examined an hour after the reaction was started.

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## BIOGRAPHICAL SKETCH

Michael Stanley Mitchell was born on the 26<sup>th</sup> of September in the year 1979. He grew up in Gurnee, IL, with his parents, Larry and Linda Mitchell, and two siblings, Greg and Cathy Mitchell. While attending Warren Township High School, he was part of the Science Olympian Team. He competed in many fields, one of which was chemistry.

After high school, he moved to Peoria, IL. There, he attended Bradley University and became interested in both chemistry and biochemistry. He worked on two different projects. One project studied P450, and the second was synthesizing different organic molecules. He received a B.S. in both biochemistry and chemistry.

While in Peoria, he worked at the United States Agricultural Laboratory. He was a technician in the polymer labs. One project attempted to make a biodegradable using starch.

Following undergraduate, he moved to Gainesville, FL, and went to the University of Florida. He obtained his master's while working for David Richardson. He studied the oxidation of biological molecules.